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Los Angeles

Modeling Rett Syndrome with Human Induced Pluripotent Stem Cells

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in Molecular Biology

by

Minori Ohashi

2017

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ABSTRACT OF THE DISSERTATION

Modeling Rett syndrome with human induced pluripotent stem cells

by

Minori Ohashi

Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2017

Professor Kathrin Plath, Chair

Rett syndrome is a neurodevelopmental disorder that predominately affects females and is one of the most common causes of intellectual disability in females. The syndrome is characterized by developmental stagnation, cognitive deficits, seizures and other autism-like symptoms. In more than 95% of cases, Rett syndrome results from de novo mutations in the gene encoding methyl CpG binding protein 2 (MECP2), which is found on the X chromosome. However, despite numerous studies implicating the loss of this protein in Rett syndrome disease pathology, the precise reason why loss of MECP2 expression causes these clinical symptoms remains unclear. To further determine the role for mutations of MECP2 in Rett syndrome, we have generated isogenic lines of human induced pluripotent stem cells (iPSCs), neural progenitor cells (NPCs), and neurons from patient fibroblasts that either express or lack MECP2 to minimize genetic variability. Our data showed the neurons derived from NPCs adopted typical morphologies regardless of

MECP2 expression, and all NPCs produced neurons and glia at the same rate. In patient iPSC-derived neurons, loss of MECP2 was correlated with Akt deactivation and reduced dendritic complexity. Molecular profiling uncovered a reduction of 5hmC, increased expression of subtelomeric genes, and shortening of telomeres in the absence of MECP2 in hiPSCs, NPCs, and neurons. Neurons made without MECP2 show signs of stress, including induction of gamma-H2aX, p53, and senescence, which are typical molecular responses to telomere shortening. The induction of p53 appeared to affect dendritic branching in Rett neurons, as p53 inhibition restored dendritic complexity. Examination of Rett patient brains uncovered similar molecular phenotypes suggesting that our disease-in-a-dish model yielded insights into authentic human Rett syndrome patient phenotypes. These data point towards a role for MECP2 in regulating telomeres and could form a molecular basis for a new understanding of the etiology of Rett syndrome.

The dissertation of Minori Ohashi is approved.

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2017

This dissertation is dedicated to
Dr. Hiroshi Ohashi, Yoshiko Ohashi

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Chapter 1: Introduction

Early embryonic development

In order to transform from a one-cell embryo into a mature organism, human development requires many complex and self-directed processes. Human prenatal development can be divided into 3 stages: germinal (wk 1-2), embryonic (wk 3-8) and fetal (wk 9-38). After fertilization in the ampulla of the oviduct, a series of mitotic divisions occurs during the first week to establish early cleavage-stage blastomeres (2-cell, 4-cell and so on) that are known to be totipotent. Blastomeres undergo compaction to form a morula in which the inner cells are segregated from the outer cells by tight junctions. At around day 5, a fluid-filled cavity (blastocoel) is generated, and the entire cell mass is called now as blastocyst. The blastocyst consists of an inner cell mass (embryoblast) and an outer cell mass (trophoblast) and implantation takes place at this stage. The embryoblast further differentiates into epiblast and hypoblast tissues that give rise to the embryo, whereas the trophoblast gives rise to the placenta. The blastocyst then further differentiates during the second week of development. The primitive streak is formed on the dorsal surface of epiblast and epiblast cells move toward primitive streak during the third week. This process of invagination is called gastrulation, and leads to the formation of the three germ layers (ectoderm, mesoderm and endoderm).

CNS formation and molecular regulation

The human nervous system is divided into the central nervous system (CNS) that includes the brain and the spinal cord, and the peripheral nervous system (PNS) that consists of the nerves that connect the CNS to the rest of the body. CNS formation begins during the third week of development as a result of gastrulation. At the tip of the primitive streak, the

primitive node appears and functions as the embryonic organizer—a structure that was first discovered by Hans Spemann in the *Xenopus* embryos¹. Ingression of epiblast cells through the node induces them to become the notochord. This structure defines the middle of the embryo and disappears after early development. The notochord then secretes antagonists of BMP signaling—including *Noggin*² and *Chordin*³ (activated by a transcription factor *Goosecoid*⁴) and *Follistatin*⁵—in order to induce the overlying ectoderm to become neural tissue. This neuroectoderm or neuroepithelia, then thickens to become the neural plate which will give rise to the entire CNS. The rest of original ectoderm is then divided into skin epidermis (directed by BMP4 signaling) and neural crest cells. While inhibition of BMP signaling directly leads to formation of forebrain and midbrain tissues, additional signaling by Wnt Family Member 3A (WNT3a), retinoic acid (RA) and fibroblast growth factor (FGF) induces caudalization of the neural tissue and leads to hindbrain and spinal cord formation. By the end of the third week, the neural plate begins to fold and eventually fuses, resulting in the formation of the neural tube. This process is referred as neurulation. The neural tube is also polarized along its dorsal-ventral axis: the notochord initially secretes Sonic hedgehog (SHH) to induce formation of the floor plate on the ventral side of the neural tube, and then the floorplate takes over SHH production in order to establish a SHH gradient along the dorsal-ventral axis of the neural tube.

At a cellular level, the neural tube consists of a single layer of pseudostratified columnar neuroepithelial cells which are considered to be neural stem cells. These cells can undergo symmetric proliferative or asymmetric neurogenic divisions to thicken the

epithelium. With the onset of neurogenesis, the neuroepithelial cells transform into radial glial cells, which are bipolar cells with astroglial features and express markers such as glial fibrillary acidic protein (GFAP), the Ca^{2+} binding protein (S100 β), vimentin and brain-lipid-binding protein (BLBP), and the astrocyte-specific glutamate transporter (GLAST)⁶. Their cell bodies remain along the lumen of the neural tube, which eventually becomes the ventricular zone that lines the lateral ventricles. Throughout their life, the radial glia cells retain their apico-basal polarity, and during neurogenesis they can continue to divide symmetrically or asymmetrically to produce neurons directly or intermediate progenitor cells⁷. The intermediate progenitor cells are multipolar cells marked by the expression of T-box transcription factor (TBR2), cut-like homeobox 1 and 2 (CUX1, CUX2) and neurogenin 2 (NGN2). They remain in subventricular zone (SVZ)—directly above the VZ—where most of their divisions take place. Intermediate progenitor cells can also generate neurons, astrocytes and oligodendrocytes.

Human cortical development

During the fourth week of human development, the neural tube extends cranially and caudally to form the three primary brain vesicles: the prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain). Our focus will remain on neurogenesis in the forebrain, particularly cerebral cortex, for the rest of this thesis. In the sixth week, the prosencephalon is further subdivided into two secondary vesicles: the telencephalon and diencephalon. The telencephalon eventually becomes the cerebral cortex, basal ganglia, amygdaloid claustrum, lamina terminalis, olfactory bulbs, and hippocampus, whereas the diencephalon becomes thalamus, hypothalamus, mammillary

bodies, neurohypophysis, pineal gland, globus pallidus, and multiple optic structures in adults.

The human cerebral cortex that is derived from the telencephalon consists of six layers. The cortex forms in an inside-out manner, where neurons made during early development make up the deepest layers (layer VI), whereas neurons made later in development form the outermost layers (layers II/III) with the exception of the layer I being born first. Neurogenesis in the neuroepithelia of the telencephalon begins as early as embryonic day 33 (E33) in humans⁸. These first neurons—called predecessor cells—migrate from the basal telencephalon and initiate formation of the preplate region located between the pia matter and the ventricular zone. The preplate also includes Cajal-Retzius cells that arise locally from the ventricular zone or elsewhere in the brain. These cells define the outermost boundary of the brain and secrete a protein called Reelin which acts as a stop signal for migrating neurons⁹. The influx of cells into the preplate then causes it to separate into the marginal zone (MZ) and the subplate (SP), and then the cortical plate (CP) forms between MZ and SP. The MZ becomes layer I whereas the CP forms layer II-VI (Figure 1).

Abnormal brain development and psychiatric disease

In the brain, the neocortex consists of 70-80% excitatory pyramidal neurons that have long projections, and 20-30% inhibitory smooth interneurons that only have short local projections¹⁰. Most of the interneurons in CNS are inhibitory although there are some types of glutamatergic excitatory interneurons. Glutamate and/or aspartate are the major

excitatory neurotransmitters while gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the cortex. Cortical excitatory glutamatergic neurons are generated within the cortex on the wall of dorsolateral telencephalon. In contrast, cortical GABAergic interneurons are produced in the medial and caudal ganglionic eminences (MGE and CGE, respectively), which are transient embryonic structures located in ventral telencephalon¹¹. In rodents, almost all the cortical interneurons originate from the ganglionic eminences and they migrate tangentially from the basal telencephalon into the cortex. They can be identified by the expression of *Dlx 1 and 2*, *Nkx 2.1* and *Lhx 6.1*. Recently, however, it has been shown that human cortical inhibitory interneurons can be generated locally in the dorsal telencephalon within the neocortex as well^{12,13}.

Anomalies in interneurons are implicated in cognitive impairment. Recent evidence reveals that deficits in the function of GABAergic interneurons early in development are not only linked to hyperexcitability and epilepsy¹⁴ but also to many neuropsychiatric disorders including schizophrenia, Angelman's syndrome, autism, Fragile X syndrome, Down syndrome, Rett syndrome and other kinds of intellectual disability (ID) syndromes¹⁵. Notably, the disease-related genes for these disorders have also been shown to be preferentially expressed in developing cortical interneurons, suggesting a causative role of improper inhibitory neurons development in their disease process¹⁶. There are many different subtypes of interneurons (>20 different classes) that are defined by the morphology and biochemical/electrophysiological characteristics of the neurons, and these subtypes are genetically specified in early development. However, the functional consequences of these subtypes in early normal human brain development remains

elusive and further studies are required to understand how disruption of these inhibitory neurons could lead to neurological dysfunction.

Intellectual disability and Rett syndrome

Approximately 1-3% of the global population is affected by some form of ID syndrome with the highest prevalence in children as a result of developmental delays¹⁷. ID is defined as showing limitations in intellectual and adaptive functioning¹⁸, and the disability usually becomes evident before 18 years of age. While both environmental and genetic factors appear to contribute to ID syndromes, the exact etiology of the majority of ID cases is unclear¹⁹. One of the most common ID syndromes to affect females is Rett Syndrome. Rett syndrome is a neurodevelopmental disorder that affects 1 in 10,000 live female births worldwide, and is generally prenatal lethal for males²⁰. Patients appear to develop normally until 6 to 18 months of age, and then begin to present with developmental stagnation, autonomic nervous system dysfunction, seizures, purposeful repetitive hand movements, gait abnormalities, sudden loss of acquired motor skills and speech, and severe cognitive deficits²¹. Clinically these symptoms, especially impairment of the autonomic nervous system, are difficult to manage with higher incidence of death in adolescence, and as of now there is no treatment available.

In more than 95% of cases, the disorder is caused by *de novo* loss-of-function mutations in the gene for methyl-CpG-binding protein 2 (MECP2) found on the X chromosome²². This is a sporadic disorder (for over 99% of cases) where most of the *de novo* mutations originate in the paternal germline²³ and patients are exclusively heterozygotes as

complete loss of MECP2 is generally prenatal lethal. During early development in normal female mammals, one X chromosome is randomly inactivated in order to achieve gene dosage compensation. This process leads to mosaic expression of MECP2 in female Rett patients, with a random distribution of cells that express the X chromosome carrying the normal or mutant allele of MECP2. The MECP2 gene consists of four exons and spans approximately 76kb on the long arm of the X chromosome (Xq28) and its sequence is highly conserved across many vertebrates. The gene generates two isoforms of the protein as a result of alternative splicing of the pre-mRNA transcript: MECP2-e1 that includes exon 1, 3, and 4, and MECP2-e2 that includes exon 2, 3, and 4. The protein structure of MECP2 is reported to be highly disordered with 60% of being unstructured²⁴. Further, MECP2 receives various post-translational modifications (PTMs) including phosphorylation, acetylation, sumoylation and ubiquitination, although the functional roles of these PTMs have yet to be fully explored.

Severity of the disease is known to be correlated with the skew of WT X chromosome inactivation and the type of mutation generated^{25,26}. Hundreds of different MECP2 mutations have been thus far reported in females,²⁷⁻³⁰ though eight mutations are found most commonly²⁵. These mutations can affect any of the four different functional domains of MECP2, including the transcriptional repression domain (TRD), the methyl-cytosine-binding domain (MBD), the NCOR–SMRT interaction domain (NID) that is located within TRD, and a carboxyl terminal domain (CTD) (Figure 2). Additionally, it has been proposed that there are AT-hook motif sequences before, within and after TRD that bind to minor groove of AT-rich DNA sequences³¹. Most mutations are missense, nonsense or

frameshift mutations in exon 3 or exon 4 where these functional domains are present, although mutations on the 5' end of the gene can also cause the disease. These mutations mostly lead to either partial or complete loss of function or greatly reduced levels of MECP2.

MECP2 appears to be critical for normal human development, as hemizygotic mutations in males often leads to death in utero. While all cells in the body express MECP2, MECP2 is most highly expressed in post-mitotic neurons in the brain³². Indeed, female patients show motor dysfunctions, purposeful hand movements, seizures and gait abnormalities. While loss of MECP2 does appear to cause reduced brain weight and circumference, neuronal size, dendritic complexity, spine density and motility, particularly in the cortex and hippocampus, there is little to no apparent neuronal degeneration or gliosis³³⁻³⁵. Furthermore, re-expression of MECP2 in Rett mouse models can reverse the neurological phenotypes, suggesting that there is no irreversible damage to neurons lacking MECP2³⁶.

The use of patient-derived iPSCs as a cellular disease model

Considerable efforts have been devoted to uncovering the disease mechanism of neurodevelopmental disorders. Thus far, much of our current knowledge of human disease has been gained by using animal models such as genetic mouse models. These models have provided invaluable insight into the function of many disease relevant genes and greatly contributed to our overall understanding of genotype/phenotype relations at the molecular level. Yet translating findings from animal studies to human disease treatment has often been unsuccessful partially due to the species-specific differences in

basic anatomy and physiology, and considerable differences between species at the genome level³⁷. This is particularly true in the brain where significant differences exist in the developmental processes of humans and rodents³⁸. For instance, mice are lissencephalic and lack a well-developed prefrontal cortex or temporal cortex³⁹. These differences are of particular importance because the larger size and surface area of the folded human brain is known to be important for our higher intellectual ability⁴⁰. The greater volume and thickness of the human cortex is also responsible for the dramatic neocortical expansion of the outer subventricular zone (OSVZ), which is lacking in rodents. Human mid-gestational corticogenesis is also distinctive in that the larger SVZ is separated into the inner subventricular zone (ISVZ) and OSVZ. In humans, the OSVZ is the major site of neurogenesis, and is largely responsible for the much greater number and density of neurons, surface area (due to the formation of sulci and gyri), and thickness of the human cortex⁴¹. The OSVZ contains highly neurogenic basal radial glial cells, which resemble apical radial glial cells (aRGC) in the VZ due to their retention of a basal process but lack of an apical process. In contrast, lissencephalic species have greatly reduced OSVZ and a very low number of basal radial glial cells (bRGC) compared to humans⁴². Furthermore, cortical inhibitory neurons in mice are exclusively derived from the ventral source whereas those in humans are derived both from the dorsal and ventral sources. Given these inherent differences, modeling brain development using rodent models is potentially limited and thus may not fully reproduce many aspects of the mechanism of human neurological disease.

For these reasons, it is critical to validate findings made in animal models and further develop these findings in a human system. However, the use of human primary cells in the field of brain research is particularly challenging due to the difficulty of obtaining primary cells from diseased donors in sufficient numbers, and the challenges associated with primary cell culture in general. Also, due to the heterogeneous nature of the brain, it is difficult to purify specific cell types such as inhibitory neurons from living patients at any stage of development. Post-mortem samples can be used as an alternative but these often represent the end stages of the disease. Therefore, a major challenge for modeling human diseases has been developing robust and predictive in vitro models that can faithfully recapitulate the disease state of a human system.

The remarkable discovery of the induced pluripotent stem cells (iPSCs)--in which human somatic cells are converted to a pluripotent state using exogenous expression of key transcription factors⁴³⁻⁴⁵--has created new possibilities to more accurately model human disease in a dish. iPSCs share many characteristics with embryonic stem cells in that they can both self-renew indefinitely and can differentiate into almost any cell lineage of three primary germ layers (endoderm, ectoderm, mesoderm). This technique enables reprogramming of a diseased patient's own fibroblasts into a pluripotent state, and then re-differentiating the cells into disease-relevant cell types. As a result, this approach provides a new avenue for producing a disease state in a dish with unlimited supply, and one that can closely reflect what is occurring in patients in vivo from the embryonic stage to terminal differentiation.

Development of iPSC lines and in vitro directed differentiation

Since the emergence of iPSCs, a number of groups have studied Rett syndrome using iPSC models. Typically, these studies used iPSC lines that either expressed MECP2 or lacked this protein and looked at differences in nuclear and soma size, neuronal differentiation, number of synapses, dendritic complexity, spine density, and electrophysiology⁴⁶⁻⁵¹. Despite the early success of these studies in showing recapitulation of patient phenotypes *in vitro*, a few key issues need to be addressed in order to more faithfully model the disease state and identify substantial phenotypes.

First, it is crucial to use well-characterized patient-derived iPSC lines, and have multiple defined subjects and controls for comparative studies in order to overcome the inherent variability of human pluripotent differentiation^{52,53} and differences in genetic background. In some published studies they directly compared iPSCs derived from patients and lines derived from healthy individuals, which fails to take into account that the observed phenotypes may not be due to the disease mutation but rather other genetic differences between the patient and control samples. Second, the success of modeling disease *in vitro* largely relies on faithful differentiation of the iPSCs. It is therefore critical to establish robust, efficient, and well-defined directed differentiation methods so as to achieve pure populations of the functional cell types implicated in disease. Third, potentially due to suboptimal culture conditions, iPSC derived neurons often represent an early immature state of neuronal development, which makes it difficult to study the pathology at later stages. It is now clear that *in vitro* cells retain intrinsic molecular clocks that closely mirror the sequence and timing of biological events *in vivo* and therefore requires prolonged and

well-optimized differentiation to achieve mature functional properties^{39,54-56}. Addressing these issues with iPSCs will be key to successfully establishing an accurate *in vitro* model and acquiring an in depth understanding of the molecular pathogenesis of Rett syndrome.

The role of MECP2 in transcriptional regulation during human brain development

MECP2 was originally identified as a methylated DNA binding protein located on the X chromosome^{57,58}, and was originally suggested to potentially affect transcription and chromatin organization. Subsequent *in vivo* studies have implicated MECP2 in the regulation of DNA methylation, the epigenome, differentiation, and basic neuronal physiology⁵⁹⁻⁶⁶. Despite growing efforts to understand the basis of the disease, the molecular and cellular functions of MECP2 remain unclear and little is known on how loss of this protein plays a role in developmental and neurological defects. As suggested by the evidence that MECP2 binds to methylated DNA, a hallmark of gene silencing, early work proposed MECP2 as a global transcriptional repressor⁶⁷. Other studies show that MECP2 forms a complex that includes histone deacetylase (HDAC) with co-repressor SIN3A or other co-repressors⁶⁸, confirming a gene silencing function of MECP2^{69,70}. Furthermore, a recent study showed that MECP2 can bind to methylated CA dinucleotides (mCA)⁷¹ and specifically repress the expression of long genes that are enriched with mCA and preferentially expressed in the brain⁷². Still, the exact role of MECP2 in transcriptional control has been controversial and debated for years. One group initially showed that loss of MECP2 leads to very few gene expression changes comparing wild-type and mutant mouse brain⁷³ and then in a separate study showed that loss of MECP2 leads to a small change in expression of all genes in a pluripotent stem

cell model⁷⁴. Another study also revealed that MECP2 can be a transcriptional activator when it binds to the co-activator cyclic AMP-responsive element-binding protein 1 (CREB1)⁷⁵. Whether MECP2 functions globally or targets specific genes has also been unclear. Therefore, further investigation of the transcriptional impact induced by loss of MECP2 is of great importance to precisely understand the role of MECP2 in gene regulation. One major challenge in defining the molecular function of MECP2 in the human brain is the cellular heterogeneity of the brain that could potentially mask MECP2-related gene alterations. The general hypothesis of the field is that MECP2 regulates transcription in cell-type and developmental-stage dependent manner. For example, *Fos* gene expression is known to be increased or decreased depending on certain structures of the brain in the *MECP2* null mice⁷⁶. Thus it is crucial to perform gene expression analysis of disease-relevant cell types in an appropriate developmental context.

The mechanism of MECP2-mediated epigenetic regulation and chromatin structure

MECP2 has also been implicated in epigenetic modulation as MECP2 binds to both 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC)^{57,77}. A previous *in vivo* study using mouse brain showed that MECP2 binds uniformly across the genome but selectively binds to methylated CG⁷⁸. 5mC is a covalent modification on DNA typically associated with transcriptionally silenced loci. However, ten-eleven translocation (TET) family enzymes can hydroxylate these marks to form 5hmC, which eventually initiates a cascade leading to demethylation and reactivation of DNA loci. While both 5mC and 5hmC are stable epigenetic marks, it appears that 5hmC is significantly enriched in the

brain compared other tissues⁷⁹, particularly near active genes in neurons⁷⁷. The formation of 5hmC was previously found to be critical for neuronal differentiation and brain development⁸⁰. Furthermore, MECP2 has been identified as a major 5hmC binding protein in the brain and has been shown to have similar affinity for 5mC and 5hmc⁷⁷. Additionally, our collaborators have observed that 5hmC distribution is altered in response to different MECP2 expression levels in their murine models⁸¹. These findings suggest that MECP2 may play a role in recognizing and interpreting the dynamics of 5mC and 5hmC and thus modulating gene expression in the brain. Having multifaceted functions, MECP2 has been previously suggested to also be involved in chromatin organization^{67,78,82-84} and alternative splicing^{85,86} by directly binding to RNA⁸⁷. Furthermore, MECP2 is reported to bind to and interact with multiple different proteins (approximately 40) including ATRX, Lamin B, DNMT1, MBD2 and TET1⁸⁸. However, the functional relevance of these interactions to the pathology of Rett Syndrome has yet to be determined.

Intellectual disability and telomeric and subtelomeric domains

An increased awareness and focus of study on ID syndromes has begun to uncover patterns shared by these disparate disorders. Evidence from a wide variety of studies suggests that 10-15% of all ID patients exhibit anomalies in subtelomeric domains of the genome (regions located adjacent to telomeres at the end of chromosomes)⁸⁹⁻⁹³. Due to their extensive homology between different chromosomes, deleterious chromosomal rearrangements including unbalanced translocation, deletion, and duplication often take place in these regions. These subtelomeric rearrangements have been repeatedly

reported to be linked to developmental delay and ID⁹⁴⁻⁹⁶. Together, these studies indicate that chromosome end regions play an important role in proper human development.

In terms of structure, telomeres are DNA-protein complexes that have tandem repeats of the TTAGGG sequences found at the end of all chromosomes. In general, human telomeres consist of 3-20kb duplex repeats in length with 3' single stranded overhang that is 100-200 nucleotide long. This overhang inserts into a duplex DNA tract, folding into a T loop that is protected and facilitated by a protein complex called shelterin^{97,98}. By maintaining a loop-like structure, it prevents the telomere from being recognized as a double stranded break and activating DNA damage response pathway or being degraded by exonuclease. Due to end replication problem during lagging strand synthesis in cell division, telomeres get progressively shorter and are therefore considered to be akin to biological clocks for cells.

The region directly adjacent to telomeres is known as the subtelomeric region of the genome. Subtelomeres are highly polymorphic and dynamic, leading to frequent recombination events. Subtelomeric domains consist of two regions: the terminal region (5-300kb) with segmental duplications and the chromosome specific regions⁹⁹. Epigenetically, both telomeres and subtelomeres are heterochromatic and are marked by HP1, H3K9me3, and H4K20me3¹⁰⁰. While telomeric methylation remains controversial given the low levels of non-CG methylation in mammals in general, subtelomeric DNAs have been shown to be heavily methylated by DNMT1 and DNMT3a/b thereby preventing frequent homologous recombination to maintain genome stability¹⁰¹. Subtelomeres are

also enriched with genes whose expression level can be modulated by adjacent telomeres, a phenomenon known as telomere position effect (TPE) or TPE over long distances (TPE-OLD)^{102,103}. Because of the large size of the telomere, it can fold back, making a loop that directly interacts with distal target genes and silences their expression. Most importantly, this effect is a telomere length dependent manner: the longer the telomere, the greater the silencing.

Telomeres themselves can be transcribed as a non-coding RNA called telomeric repeat-containing RNA (TERRA)¹⁰⁴. TERRA is transcribed from subtelomeric sequences into telomere repeats and directly interacts with MECP2¹⁰⁵. Because it codes for telomere repeats, the only known function for TERRA is to inhibit the TERT enzyme complex that can lengthen telomeres by competing with its RNA primer¹⁰⁶. Evidence suggests that increased TERRA expression is well correlated with short or damaged telomeres¹⁰⁷⁻¹⁰⁹. Both upregulation of TERRA and shorter telomeres have been also reported in developmental disorders¹¹⁰. Yet, it is unclear that which of these antagonistic processes lead to the changes first: short or damaged telomeres could induce upregulation of TERRA due to loss of the TPE, or TERRA could initiate the telomere shortening by inhibiting telomerase. Regardless, the underlying mechanism of how the dysfunction of telomeres and subtelomeric regions leads to abnormal brain development remains to be discovered.

Taken together, these findings from various studies led us to ask whether MECP2 has an inherent role in the regulation and dysregulation of telomeric and subtelomeric domains

of chromosomes, and loss of this regulation results in the neuropsychiatric abnormalities and severe ID of Rett Syndrome. To further uncover potential molecular and cellular functions in these domains, in the following chapters we describe a study that focuses on the mechanisms by which methylation, subtelomeric genes and telomere dysfunction are regulated by MECP2.

In Chapter 2, we begin by establishing *in vitro* model using patient-derived iPSCs and characterizing disease-relevant phenotypes. Particularly, we are interested in deriving inhibitory GABA releasing interneurons, as lack of MECP2 in these neurons lead to most severe neurological phenotypes compared to other neuronal types¹¹¹. Using our established protocols, this approach enables a dissection of the role that MECP2 plays in specific neuronal subtypes in Rett syndrome. In Chapter 3, using defined cell types of CNS, we pursue in integrative analysis of transcriptomes and methylomes and investigate whether there are any anomalies particularly in subtelomeric and telomeric domains. Given the discrepancies that exist in the reported effects of MECP2 loss on gene expression patterns, we also aim to further define the transcriptional impact induced by loss of MECP2 to understand its role in gene regulation in specific cell types during development. Additionally, to elucidate whether MECP2 plays a role in affecting methylation, we profile genome-wide 5hmC distributions in cells with or without MECP2. The overall abundance of 5hmC and alterations at certain genomic loci are to be analyzed. By comparing the 5hmC patterns, we gain valuable mechanistic insights on the role for MECP2 in regulation of 5hmC. The overall goals of this proposed study are to define a precise molecular role for MECP2 in genetic and epigenetic regulation and

uncover its inherent role in subtelomeric domains. These analyses will improve our understanding of the disease process of Rett syndrome, and may serve as a foundation for developing effective therapeutic strategies.

Figures

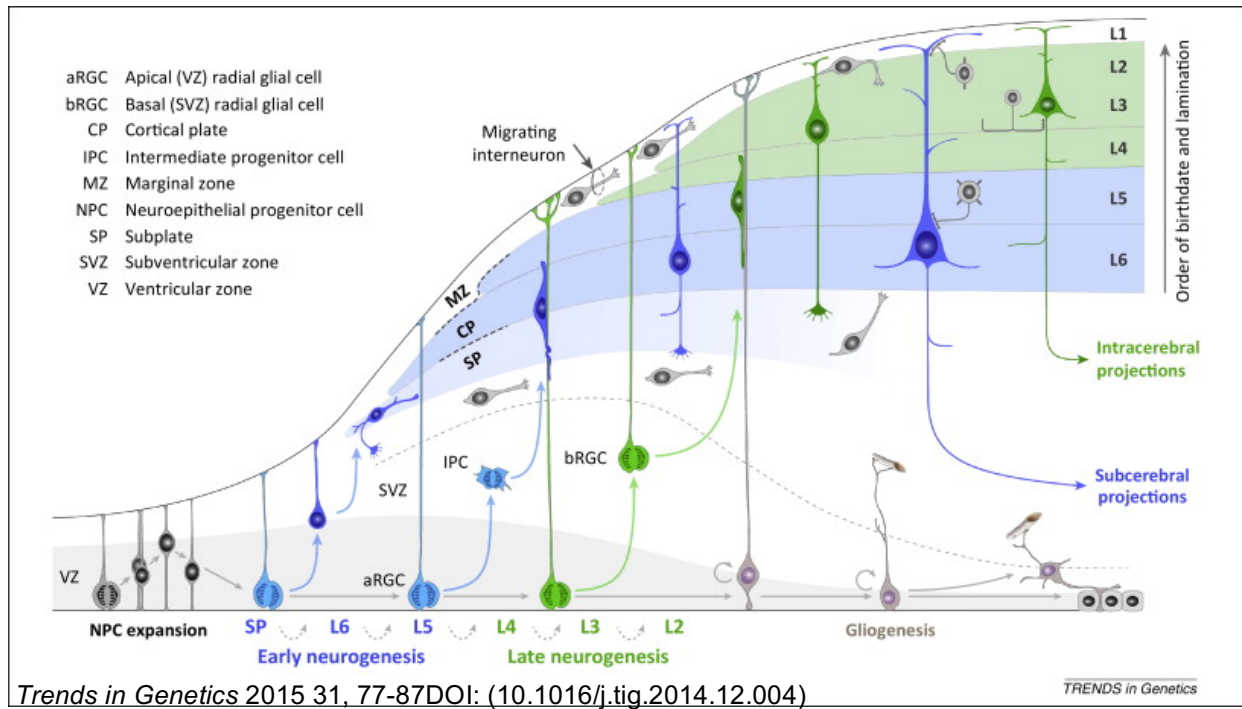
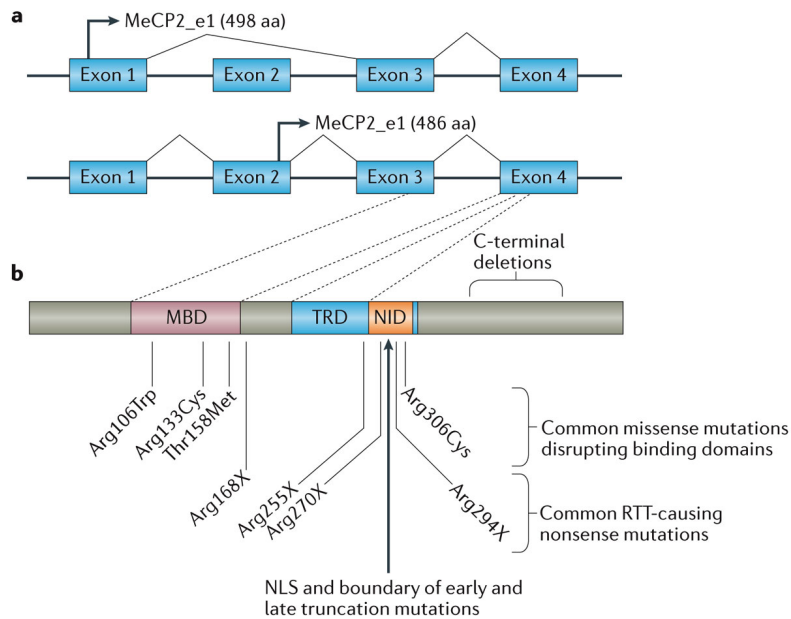


Figure 1-1: Early and late neurogenesis in the cortical development

Schematic representation of the process of corticogenesis during neurodevelopment.



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Figure 1-2: MECP2 gene

Schematic representation of MECP2 gene shows the structure, domains and isoforms of MECP2.

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Chapter 2: Rett syndrome in a dish

Introduction

Rett syndrome is a monogenic neurodevelopmental disorder that typically affects females, leading to autistic spectrum characteristics, growth arrest, seizures and microcephaly. While the disease is caused by loss-of-function mutations in MECP2 on the X chromosome, little is known about how the loss of this protein plays a role in neurological defects in the brain. Much of current knowledge on intellectual syndromes has been derived through the study of animal models, which in some cases do not fully reproduce human symptoms. Human PSCs hold great promise for understanding human development due to their unique capacity to differentiate into most human cell lineages in the body. The emergence of patient-derived iPSC technology has created possibilities to model the formation of human disease-relevant cells during development and faithfully recapitulate disease phenotypes in a dish. Previously, our group demonstrated that iPSC derived cells reflect their *in vivo* counterparts during early human development. In this study, we hypothesize that the molecular phenotypes of Rett syndrome appear very early in the fetal development as a result of a developmental arrest or failure.

Recapitulation of disease phenotypes *in vitro* is the most difficult aspect of disease-in-a-dish studies because of the basic differences between *in vitro* and *in vivo* environments, the inherent variability of human pluripotent differentiation¹, and potentially because of the progeny of hiPSCs appear to represent very early stages of fetal development instead of adult cell types². Much of the variability of hPSC differentiation has been ascribed to differences in genetic background³. This variability can be eliminated in the study of X-linked diseases in females because of a general lack of reactivation of the X-chromosome

during human reprogramming⁴. As MECP2 is located on the X-chromosome, some have performed disease in a dish studies with fibroblasts taken from female patients with the disease. Typically, these studies focused on one or two cell lines that either expressed MECP2 or lacked this protein and found differences in nuclear/soma size, neuronal differentiation, and electrophysiology⁵⁻⁹. In addition, some studies found similar phenotypes even when isolating mixed pools of reprogrammed cells, where essentially half the cells in culture lacked MECP2, compared to unrelated WT lines¹⁰⁻¹³. Taken together, it appeared as though clinical aspects of Rett syndrome could be recapitulated *in vitro*.

In the current study, we sought to mitigate the effect of genetic background and variability of differentiation by taking advantage of several isogenic lines of hiPSCs that either express the WT allele or the mutant allele leading to cells that express or lack MECP2⁴. In addition, several lines were made and analyzed in each category to avoid variance in differentiation potential amongst isogenic lines. Furthermore, isogenic lines were made from two independent patients with different mutations to highlight only those phenotypes associated with loss of MECP2 expression and not genetic background or variance in hPSC differentiation. In comparing multiple lines of cells, it is clear from our data that variability of differentiation still exists, despite controlling for genetic background. However, we were able to find physiological phenotypes that were consistent with loss of MECP2 *in vitro* when taking patient-derived cells through a pluripotent intermediate. Together, these data suggest that the design of the disease-in-a-dish model can have a profound influence on the ability to model disease phenotypes.

Results

To determine how loss of MECP2 expression leads to defects in the nervous system we generated a disease-in-a-dish model using induced pluripotency. Cognizant of the fact that differentiation from human pluripotent stem cells is highly variable across individual lines, culture conditions, and time, we developed an isogenic model to study Rett syndrome *in vitro* to remove the confound of genetic backgrounds. Because female patients with Rett Syndrome are usually heterozygous for mutant alleles, fibroblasts isolated from these patients display a mosaic pattern where roughly half the cells express either the mutant or WT allele. This is shown in Figure 1A, where fibroblasts were isolated from two patients with different mutant alleles, and roughly half the cells express MECP2 while the other half lack significant amounts of this protein due to destabilization of mutant transcript (R982 and R567). One of these mutant alleles is predicted to lead to a premature stop codon, while the other leads to failed transcriptional termination. Reprogramming to the pluripotent state using defined transcription factors has been shown to generally happen at the clonal level, such that individual reprogramming events in single fibroblasts generate isolated hiPSC clones. Therefore, reprogramming of mosaic fibroblast cultures from two different patients generated single hiPSC clones that either expressed MECP2 protein or lacked it (Fig 1B) (Method described in the previous study¹⁴). In addition, our work and that of others has shown that under standard conditions, the inactive X chromosome in human fibroblasts does not reactivate upon reprogramming to the pluripotent state^{4,14,15}, which is distinct from murine reprogramming¹⁶.

Thus, we were able to create multiple lines of hiPSCs with and without MECP2 from individual patients and thereby control for differences in genetic background (shown are clones made from patient 982, clones from 567 look similar). The hiPSCs generated from fibroblasts of both patients appeared to be unaffected by the lack of MECP2, expressed all appropriate markers, and successfully generated teratomas upon injection into the testes of immunocompromised mice, consistent with previous hiPSC models for loss of MECP2 (Fig S1)^{7,12,17,18}. Lack of MECP2 in patient-derived cells and specificity of antibody was also confirmed by western blot (Fig 2A). Importantly, we never observed reactivation of the silenced X chromosome that would have resulted in re-expression of the WT allele of MECP2 in any cultures regardless of differentiation status or passage. This is consistent with previous data showing that despite evidence for erosion of isolated portions of the silenced X chromosome¹⁹, the portion containing the MECP2 locus was not affected by reprogramming or differentiation.

As Rett Syndrome primarily afflicts the nervous system and MECP2 is most highly expressed in the nervous system, we first generated neural progenitor cells from all of the hiPSCs lines generated by standard protocols². Across at least two lines per patient with and without MECP2, we measured the rate of neuralization, the morphology of NPCs, and expression of typical marker genes. We were unable to detect consistent differences in these properties between multiple clones of both WT and MECP2- lines derived from both patients (Fig 1C and 2B). Furthermore, the growth rate of NPCs with and without MECP2 was not consistently different in NPCs made from either patient (Fig 2C). Next, the NPCs were further differentiated by a non-directed differentiation approach that yields

both neurons and glia (growth factor withdrawal²⁰) (Fig 1D). Both the neurons and glia made from NPCs adopted typical morphologies regardless of MECP2 expression, and all NPCs from both patients produced neurons and glia at the same rate (Fig 2D and 2E).

Various studies have shown that differences in size of nuclei or soma when comparing WT and MECP2 mutant neurons¹⁰. After extensive study and quantification across three lines from each WT and mutant, we were unable to find consistent differences in nuclear size in either NPCs or neurons that correlated with loss of MECP2. Instead, we found significant variation of nuclear size across all lines, across isogenic lines made from two distinct patients, and even across replicate experiments (Fig 2F).

Previous studies have also shown that loss of MECP2 in neurons can lead to a decrease in AKT signaling¹⁸. A similar pattern was observed here in mutant neurons generated from Rett patient hiPSCs as measured by phosphorylation of AKT and S6, while hiPSCs themselves did not seem to be affected by loss of MECP2 (Fig 1E). Dendritic complexity has been shown extensively to be reliant on MECP2 expression in various models of Rett syndrome, and we found a statistically significant decrease in complexity in neurons made in the absence of MECP2 by Sholl assay (Fig 1F). In addition, we observed qualitative differences in basic neuronal morphology between WT and mutant neurons, where the neurons lacking MECP2 had shorter, thicker processes, and their soma was not as well defined.

Previous reports implicated MECP2 in the regulation of genes that are induced in neurons in response to sustained synaptic activity through activation of pathways such as Creb²¹⁻²⁵. It was shown that phosphorylation of Mecp2 was important for its regulation and interaction at loci that are turned on by activated by Creb, such as Bdnf, C-fos, and Npas4 in murine neurons^{24,26-28}. We first assayed for CREB responsiveness in neurons by treating cultures with KCL to induce depolarization and measured phosphorylation of CREB with a phospho-specific antibody to assess its activation state. It was clear that neurons with and without MECP2 all seemed to respond similarly to KCL induction (Fig 3A and 3B). Furthermore, RT-PCR for several genes known to be induced under such conditions showed little correlation with MECP2 expression (C-FOS, BDNF and NPAS4), with essentially all lines responding similarly (Fig 3C). In all, we did not observe changes to CREB activation or responsiveness in the absence of MECP2.

Furthermore, we assayed whether the presence of MECP2 determines what types of neurons are generated in this *in vitro* model system with spontaneous terminal differentiation by growth factor withdrawal. By first looking at general neuronal and glial markers by microarray profiling, we paired samples based on presence or absence of MECP2. The relative ratios of neuronal versus glial markers and did not detect significant differences (Fig 4A), consistent with quantification by immunostaining (Fig 2D and 2E). Probing the data for markers of neuronal specification, the data generally pointed towards the neurons being generated by growth factor withdrawal as telencephalic gabaergic inhibitory neurons (interneurons) (Fig 4B). This pattern of differentiation did not change in neuronal cultures from Rett patient hiPSCs, further indicating a lack of effect of MECP2

on neurodevelopmental fate decisions in this setting. Taken together, our model system for the loss of MECP2 appears to recapitulate some (Akt activation, dendritic complexity), but not all (neuronal size, CREB responsiveness), previously reported phenotypes.

Discussion

The data from our isogenic disease-in-a-dish model was predictive for certain phenotypes found *in vivo*, in postnatal brain, or *in vitro* in previous studies. Our *in vitro* model shows significant dysregulation of Akt activation as well as reduced dendritic complexity in neurons, which was consistent with the previous observations made in another pluripotent model¹⁸. The findings on NPCs were also consistent in that the presence or absence of MECP2 did not affect differentiation potential (comparable neuralization efficiency and neuronal differentiation) or cellular phenotypes (similar morphology, levels of NPC markers and nuclear size). There were more discrepancies at neuronal stage as we did not observe any changes in nuclear/soma size or CREB responsiveness, which could be due to several possible reasons. The great variability we observed in nuclear/soma size may be in part due to our suboptimal directed differentiation conditions that resulted in heterogeneous populations of cells in general. This heterogeneity could cloud any MECP2 mediated effects on soma size and CREB signaling if the phenotype is more specific or dramatic in neurons. It is also possible that that loss of MECP2 impacts cellular phenotypes differently depending on the subtypes of neurons or developmental stages. The other possible explanation for these discrepancies is that the neurons we derived were reflected a very young state due to the fact that neuronal maturation *in vitro* requires the same amount of time as *in vivo* maturation, and therefore may have been too functionally immature to show significant differences in these measurements as *in vitro*. Finally, our isogenic system for loss of MECP2 involves a chronic loss of MECP2 where cells are created without this protein, so perhaps some other mechanism was able to compensate for its loss over time and restore its critical functions, as previously described

in a different study²⁹. Indeed, several studies have demonstrated significant discrepancies between the severity of the phenotypes due to acute loss of the gene using knock down models versus chronic genetic deletion in knock-out models. It is therefore plausible that genetic compensation could occur in our chronic model where the deleterious mutation may have been tolerated and potentially compensated for throughout development.

Nevertheless, our in vitro system did adequately recapitulate many of the human disease phenotypes. Our finding that dendritic anomalies were present even at early stages of neuronal development suggests that molecular and cellular defects could be present even before clinical manifestations appear. This emphasizes the need to study the disease beginning during the pre-symptomatic phase to further decipher how normal developmental processes go wrong in the absence of MECP2. Interestingly, dendritic abnormalities are a hallmark for cognitive deficits. Indeed, it has been consistently observed in many other ID syndromes in both post-mortem human brains and mouse models of diseases such as Down syndrome, Fragile X syndromes, Rubinstein–Taybi syndrome and Williams Syndrome³⁰. Generally, normal dendrites have a highly branched structure and the level of complexity is related to a number of inputs that neurons receive and process for higher function³¹. Dendrites are also highly plastic and can dramatically change their structure in response to external stimuli and experience, which is considered to be the basis for learning and memory³². Taken together, these studies suggest that structural anomalies seen in dendrites are a key contributing factor to pathophysiology of ID. While ID is an extremely diverse group of disorders and is caused by many genetic

and environmental factors, these different triggers may converge into the common pathological pathway to ultimately influence cognition.

To further refine our approach beyond previous iPSC models, we can use a more robust directed differentiation protocol that was recently published that specifically generates cortical interneurons by employing dual-SMAD inhibition as well as Wnt signaling inhibition. This method allows us to establish purer populations of specific neuronal subtypes, which in turn allows us to more carefully probe the molecular and cellular changes that occur across development.

While this study was able to confirm and further elucidate some of the changes that occur in the neuronal lineage upon loss of MECP2, several more lines of investigation remain to be explored. The functional differences between more mature wild-type and diseased cells could be compared by measuring electrophysiological activity and calcium signaling. To uncover potential developmental-stage-specific effects of MECP2, one could use gene-editing tools such as clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) to study the effects of acute loss of MECP2 in iPSCs, NPCs and neurons. To further examine the root of other disease phenotypes such as microcephaly, 3D *in vitro* models such as organoids—which better model the structure and complex connectivity of the brain—could prove useful. In particular, it would be extremely useful to use Rett Patient derived iPSCs to generate organoids that contain either excitatory cortical neurons or MGE-derived inhibitory interneurons and then dock the two organoids together in order to study the migration patterns of the two cell types

and how they interact in the cortex. Overall, these refined approaches will help further address the technical challenges facing in our *in vitro* model and facilitate further understanding of the disease mechanisms in Rett syndrome.

Figures

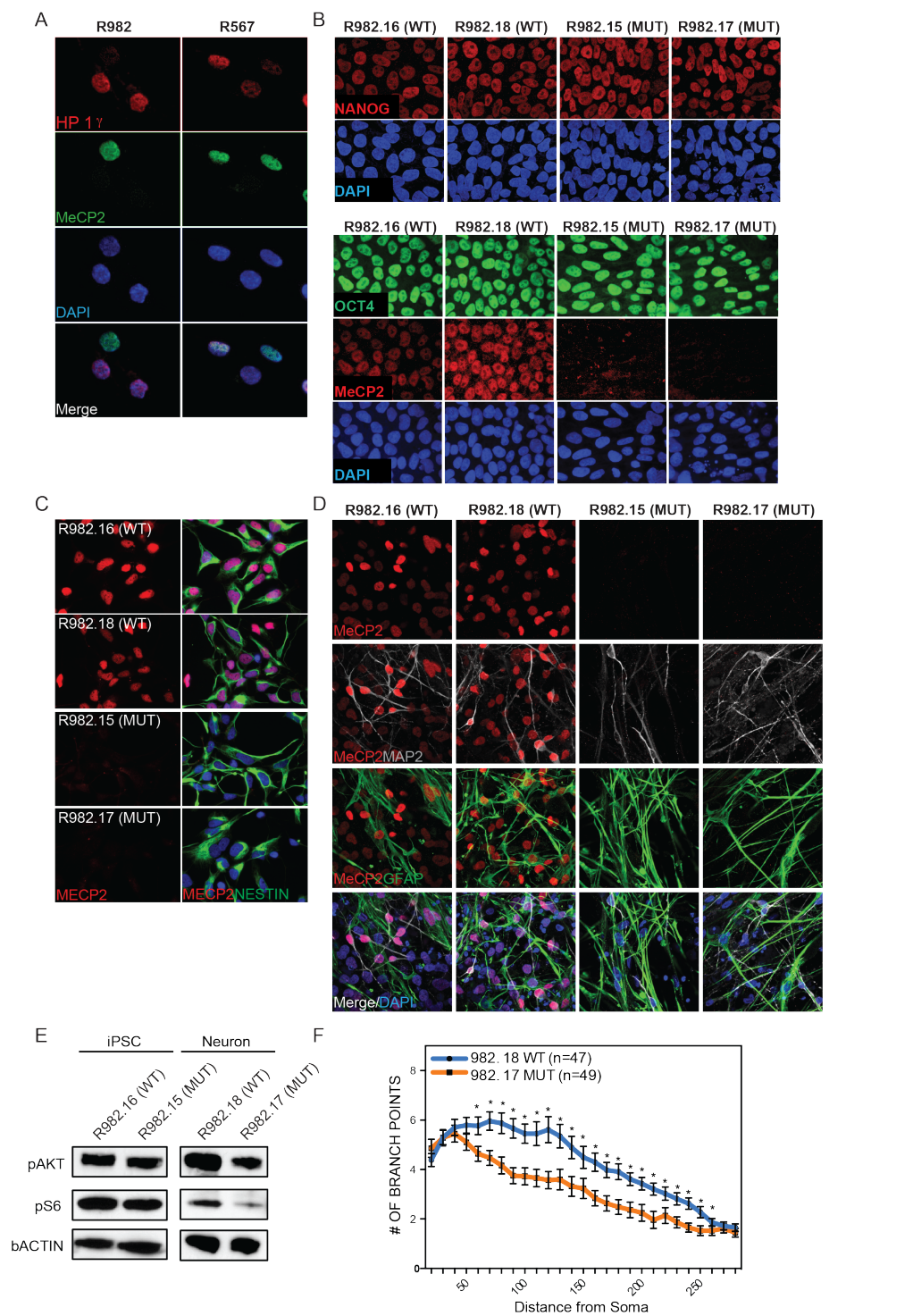


Figure 2-1. Generation of isogenic model of Rett Syndrome *in vitro*

A, Fibroblasts isolated from Rett Syndrome patients (R982 and R567) heterozygous for MECP2 mutations exhibit a mosaic pattern of MECP2 expression due to random XCI. Note that roughly 50% of fibroblasts from each patient express MECP2. B, Multiple isogenic hiPSC lines were produced from patient 982 with a typical Yamanaka protocol yielding individual isogenic clones with and without MECP2 expression from the same patient, as judged by NANOG and OCT4 staining. C, Specification of 982 patient derived hiPSCs towards neural progenitor cells yielded homogenous cultures of NPCs with and without MECP2. D, terminal differentiation of 982 patient derived NPCs towards neurons and glial by growth factor withdrawal yielded normal neural derivatives as measured by immunostaining for MAP2 and GFAP. E, MECP2⁺ and MECP2⁻ hiPSCs and neurons were generated from patient 982 (R982.16 and R982.15) and assayed for activity of the AKT pathway by western blot with antibodies that recognize the active forms of Akt and its downstream target S6. F, Sholl assay of dendritic complexity was performed on WT vs MUT neurons derived from patient 982. Increased # of branch points indicates increased dendritic complexity, measured as a function of distance from the cell body. *p value < 0.05 according to student's t test. Bar graphs represent mean +/- SEM.

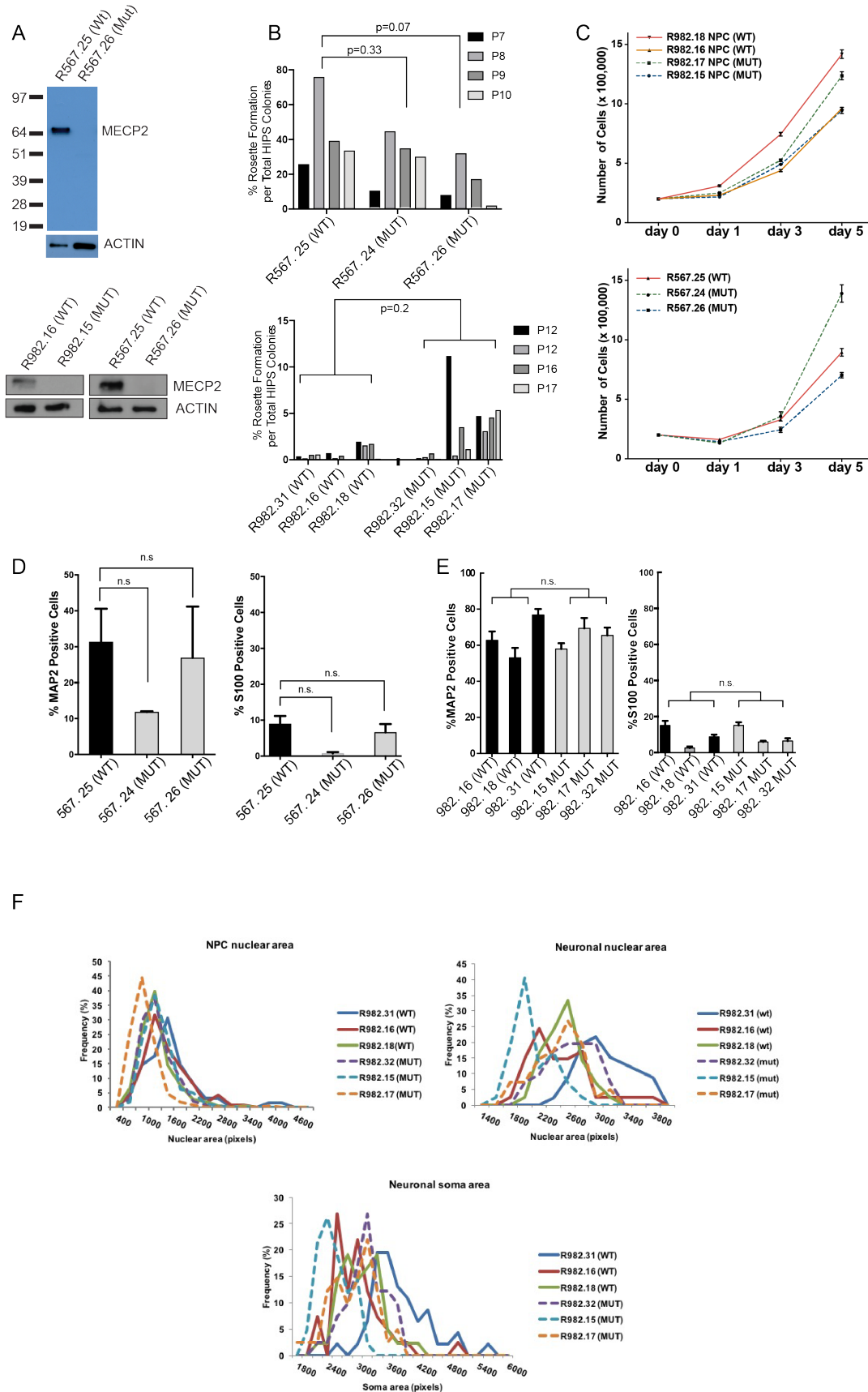


Figure 2-2. Similarity of NPCs generated with and without MECP2

A, NPCs were produced from isogenic hiPSCs of Rett patient, and assessed by western blot to validate loss of MECP2 and specificity of antibody. Top panel shows that the antibody only recognizes MECP2. Bottom panel shows that in NPCs from both patients, individual clones either express or lack MECP2. B, The ability of hiPSCs to generate NPCs was assayed in Rosette formation assay. Lack of MECP2 did not affect rosette formation across multiple lines from both patients. N=4 independent experiments. *p value < 0.05 according to student's t test (for patient R567) or ANOVA (for patient R982). Bar graphs represent mean +/- SEM. C, Growth curves show that loss of MECP2 does not affect proliferation of NPCs made from either patient. D, 3 weeks of growth factor withdrawal drives NPCs to differentiate into neurons and glia as measured here by immunostaining for MAP2/Tuj1 or S100/GFAP in patient 567 derived cultures. There was no consistent difference in differentiation potential across lines from either patient. N=2 independent experiments. Bar graphs represent mean +/- SEM. E, Patient 982 derived cultures also do not show dramatic differences in the presence of neurons or astrocytes as measured by MAP2 and S100. N=3 independent experiments. Bar graphs represent mean +/- SEM. F., Morphological measurements along with co-staining for DAPI and neuronal markers allowed for quantification of nuclear and soma size between WT and Mutant lines. Note that no statistical significance was observed for differences between WT and mutant NPCs or neurons.

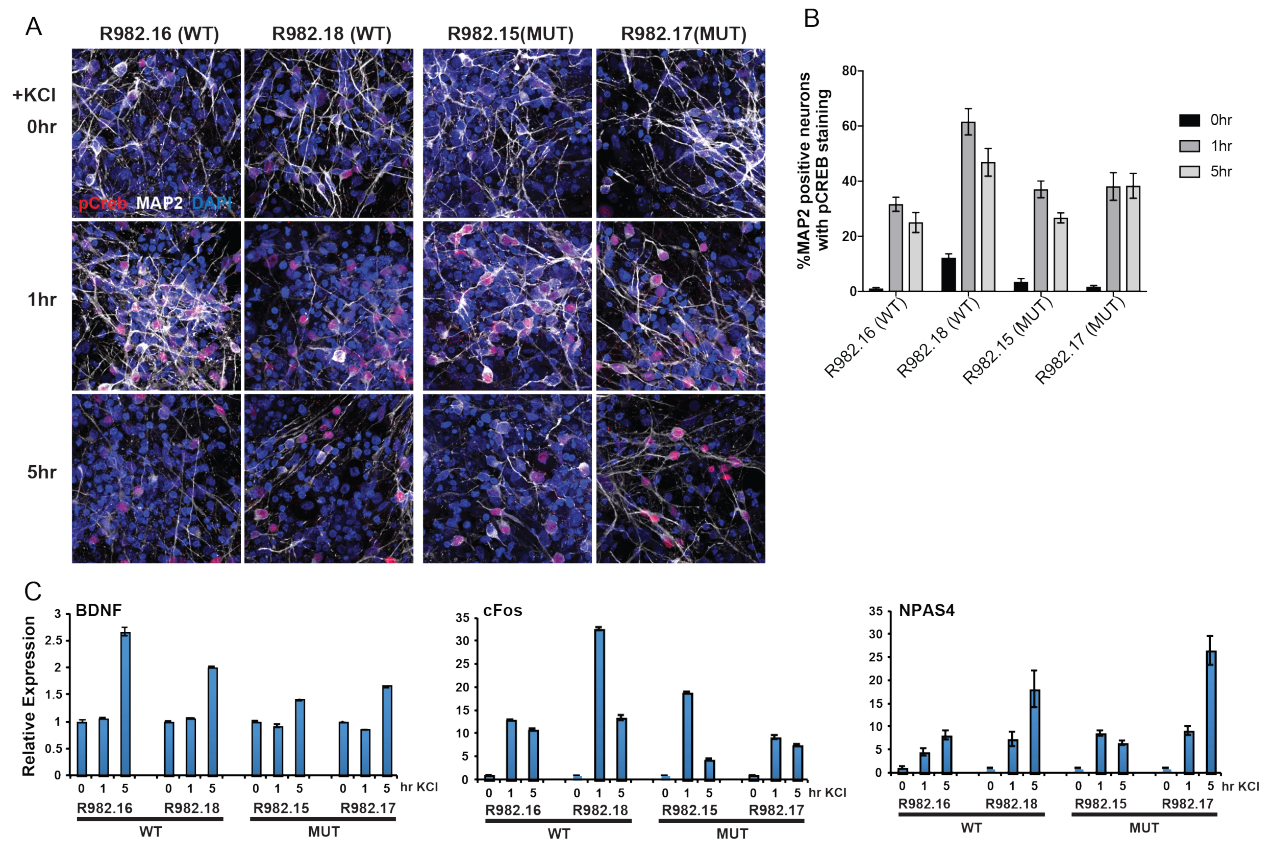


Figure 2-3. Analysis of CREB activity in MECP2 WT and MUT cells

A, Staining for phosphorylated Creb as an assay for neuronal activation by depolarization shows a similar response between neurons with and without MECP2 at 1 and 5 hours post induction by KCL treatment. B, Quantification of pCREB response to KCL treatment. C, RT-PCR for three genes well-known to be induced by depolarization shows no consistent difference between WT and Mutant neuronal cultures.

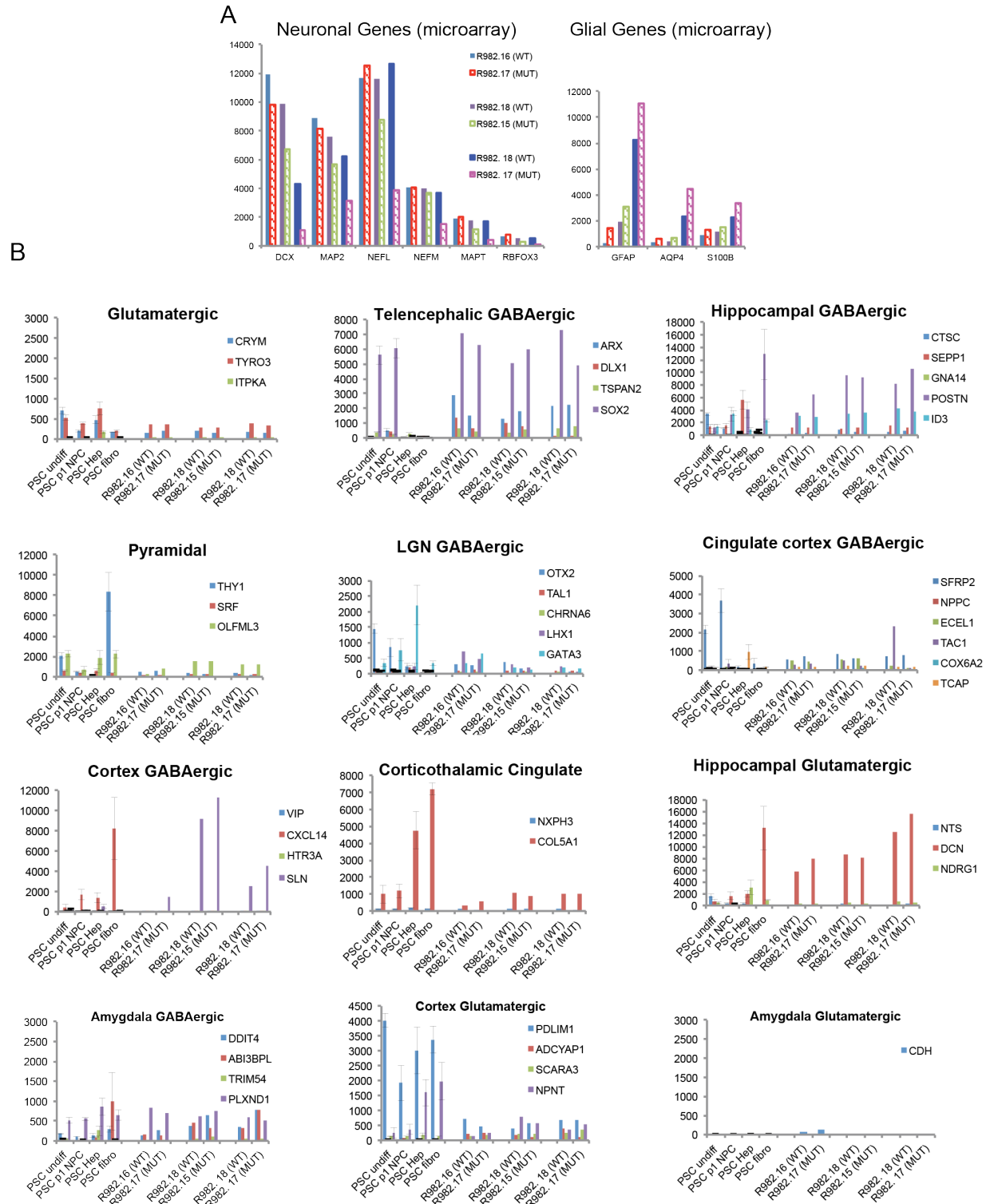
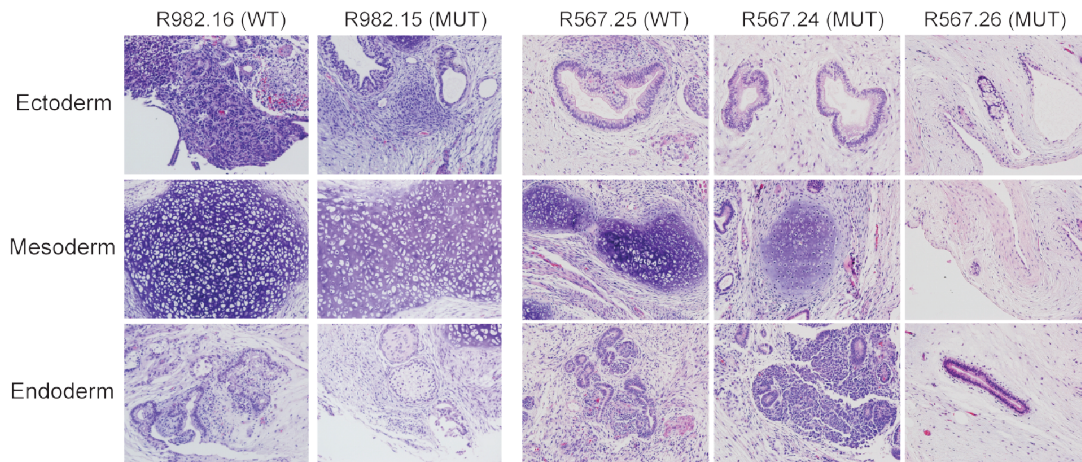


Figure 2-4. Assessing neuronal differentiation in patient derived cells by gene expression profiling

A, Gene expression profiling by microarray on neuronal cultures and focusing on just pan-neuronal or pan-glial markers suggests that there is no dramatic difference in the % of neurons or glia present despite the absence of MECP2 in several lines. B, Profiling for specifically markers of various types of neuronal markers showed that all cultures produced mostly gabaergic interneurons, regardless of MECP2 status.

Supplementary Figures



Supplementary Figure 2-1. hiPSCs lacking MECP2 are pluripotent

Teratoma assay was performed to establish pluripotency of hiPSCs made from Rett patient fibroblasts. The resulting tumors each showed evidence of differentiation towards all three embryonic germ layers.

Materials and Methods

Generation of isogenic Rett Syndrome iPSCs

Two primary fibroblast lines GM17567 (1461A>G in the gene encoding methyl-CpG binding protein 2 (MECP2)), and GM07982 (frameshift mutation, 705delG, in the gene encoding methyl-CpG binding protein 2 (MECP2)), from patients with Rett Syndrome were obtained from Coriell Cell Repositories. 1×10^5 fibroblasts were plated in a gelatin coated well of a 6 well plate in MEF media (DMEM/F12 + 10% FBS). After 8-12 hours, the cells were infected with reprogramming lentivirus that harbors polycistronic human Yamanaka factors (Oct4, Klf4, Sox2, cMyc) in DMEM medium containing 10ug/ml of polybrene and incubated overnight at 37°C in 5% CO₂ incubator. The following day, the viral media was aspirated, replaced with MEF media and cultured for 3 additional days. Cells were re-plated on the 5th day onto irradiated MEFs in MEF media. On day 6, the culturing media was changed to human ES media containing DMEM/F12 supplemented with L-glutamine, nonessential amino acids (NEAA), penicillin-streptomycin, knockout serum replacement (Invitrogen), and 10 ng/ml basic FGF. Cells were cultured in hiPSC media until iPSC-like colonies were formed. Reprogrammed colonies were further identified by live immunofluorescence staining with TRA-1-81 (Chemicon) then mechanically isolated. Individual colonies were isolated and maintained for at least 2 passages before genotyping analysis. For early passages, the iPSCs were propagated mechanically, whereas collagenase was used for subsequent passaging. hiPSCs were cultured as described previously in accordance with the UCLA ESCRO.

Generation of teratomas

Generation of teratoma was previously described (Lindgren AG, Natsuhara K, Tian E, Vincent JJ, Li X, Jiao J, Wu H, Banerjee U, Clark AT. Loss of Pten causes tumor initiation following differentiation of murine pluripotent stem cells due to failed repression of Nanog. PLoS One. 2011 Jan 27;6(1):e16478.) Briefly, a single incision was made in the peritoneal cavity of adult SCID mice and the testis was explanted through the incision site. Approximately 60,000 iPSC in a volume of 50 µl 0.5X Matrigel (BD) were transplanted into the testis using a 27-gauge needle. Four to six weeks after surgery, mice were euthanized and the tumors removed for histology. Surgery was performed following Institutional Approval for Appropriate Care and use of Laboratory animals by the UCLA Institutional Animal Care and Use Committee (Chancellor's Animal Research Committee (ARC)).

Differentiation in vitro

Neural specification with neural rosette derivation, neuroprogenitor (NPC) purification, and further differentiation to neurons and glia were performed as described previously^{2,33,34}. Relative neuralization efficiency was analyzed by counting the number of neural rosette containing colonies over total number of iPSC colonies. 6-12 35 mm wells were analyzed over four separate experiments. The proliferation efficiency of NPCs was determined by at days 1, 3, and 5 by the total number of cells present in 35mm wells seeded at 200,000 cells on day 0. The cells were detached from the plates using

accutase (Millipore) then total number of cells per well analyzed using Z1 Coulter particle counter (Beckman Coulter).

For spontaneous terminal neuronal differentiation by growth factor withdrawal, NPC cultures were subjected to growth factor withdrawal (removal of EGF and FGF) and cultured in basic medium (DMEMF12 + N2 + B27) with three quarter exchange of media every three days. Cells were cultured up to 20 weeks. Neural differentiation efficiency was analyzed four weeks after growth factor withdrawal by counting the number of cells positive for neuronal markers (MAP2 and Tuj1) over the total number of cells visualized by DAPI. NPCs were transfected with DCX-GFP reporter one day prior to differentiation using Lipofectamine 2000 (Invitrogen). Sholl analysis of DCX-GFP positive neuronal neuritis were also measured using ImageJ. All data values were presented as mean $\pm$ SEM. Student's t-tests were applied to data with two groups. ANOVA analyses were used for comparisons of data with greater than two groups.

Neuronal activation

8 weeks *in vitro* differentiated neuronal culture were subjected depolarizing stimulation with 55mM of KCl in basic media for 0hr, 1hr, 5hr and 7hr then isolated for RNA analysis and coverslips fixed with 4% paraformaldehyde for immunostaining.

RNA expression profiling

RNA purification was performed with Qiagen RNeasy kit following the manufactures' instruction. Library preparation and sequencing was performed as described. Reverse

transcription, and real-time PCR were performed as described³⁵. Microarray profiling was performed with Affymetrix Human HG-U133 2.0 Plus arrays as described³⁵.

Immunostaining and western blot

Cells on coverslips were washed with PBS, fixed in 4% paraformaldehyde for 15 min at room temperature, blocked for 1 hour at room temperature with 10% serum and 0.1% Triton-X-100, then incubated overnight at 4 °C with primary antibodies. Following primary antibody incubation, the coverslips were incubated with Alexa Fluor secondary antibodies (Invitrogen) at room temperature for 1 h and mounted in Prolong Gold with DAPI (Invitrogen). Imaging was performed on Zeiss Axio Imager A1 or Zeiss LSM780 confocal microscope. Antibodies used include the following: mouse anti-OCT3/4 (1:100, Santa Cruz Biotechnology Inc.), rabbit anti-SOX2 (1:300, Cell Signaling Technology), rabbit anti-Nanog (1:100, Cell Signaling Technology), mouse anti-Tra-1-81 (1:250, Chemicon), mouse anti-NESTIN (1:1000, Neuromics), mouse anti-MAP2 (1:500, Abcam), chicken anti-GFAP (1:2000 Abcam). Western blot analysis was performed using standard procedures as described³⁶.

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Chapter 3: Loss of MECP2 leads to telomere dysfunction and neuronal stress

Introduction

Rett syndrome is a disease associated with loss of function mutations in the gene MECP2, which was originally identified as encoding a methylated DNA binding protein¹. There are a plethora of studies in murine models demonstrating that MECP2 binds methylated DNA (both 5mC and 5hmC)²⁻⁵, while less is known about how MECP2 acts in human cells. Patients with Rett syndrome present with not only neurological deficits, but also a shorter stature and relatively small brains, consistent with the idea that the MECP2 protein plays a key role in various aspects of the basic cell biology of neurons and other cell types^{6,7}. As MECP2 is highly more expressed in the brain tissues, the consequences of loss of MECP2 particularly in various types of neurons has been explored in great detail^{1,8-11}. Despite all these data, it is still unclear how mutations in MECP2 lead to phenotypes in the human patients.

At a molecular level, MECP2 has been functionally implicated in transcriptional regulation as it binds to 5mC and 5hmC^{3-5,8,12-16}, however, discrepancies exist in the reported effects of MECP2 loss on gene expression patterns. As a result, it is debated whether MECP2 mechanistically functions as a transcriptional activator or repressor, and whether its actions are global or gene-specific in the brain. Additionally, although MECP2 has been proposed to be the major 5hmC binding protein in the brain⁵, it remains unclear how MECP2 interprets 5mC and 5hmC patterns and little is known about the molecular impact of loss of MECP2 in cytosine modification as well as its relationship with gene expression in a human model. Thus, it is of great importance to clarify the role of MECP2 in gene expression and to elucidate its underlying mechanism in epigenetic regulation.

In this study, we took advantage of several isogenic lines of patient-derived iPSCs that express either the wild-type or mutant MECP2 allele, leading to cells that express or lack the protein. In comparing multiple lines of cells, it is clear from our data that loss of MECP2 leads to profound molecular alterations specifically towards the ends of chromosomes due to a decrease of 5-hydroxymethylation, induction of subtelomeric gene expression, and shortening of telomeres. The telomere defects that arise in neurons appear to be related to defects in dendritic branching that are a hallmark of the patient disease. Together our data suggest that MECP2 may have an inherent role in regulating the end of chromosomes.

Results

Loss of MECP2 leads to disruption of hydroxymethylation of DNA

Because MECP2 is a well-established methylated DNA binding protein particularly for 5-hydroxymethylcytosine (5hmC)⁵, we first analyzed patterns of this mark across the genome in the presence or absence of MECP2 with Methylation-dependent Immunoprecipitation (MEDIP)¹⁷⁻²⁰ in hiPSCs. We used a stringent criterion to identify differentially hydroxymethylated regions (DhmRs), whereby the indicated regions had to differ by 0.2 per million reads per base pair (Fig 1A). We still observed a large number of differentially hydroxymethylated regions (DhmRs) due to the loss of MECP2 in two clones each from two independent patients (982.15 and 982.17 vs 982.16 and 982.18; 567.24 and 567.26 vs 567.25).

The loss of MECP2 led to many more hypomethylated regions than hypermethylated regions, a strong bias that indicated that MECP2 somehow promotes or stabilizes hydroxymethylation (Fig 1B). This hypomethylation is more clearly identified by plotting the Delta Methylation between the WT and MUT clones from both patients. Both patients showed a dramatic shift towards loss of methylation across both clones (Fig 1B). Mapping 5hmC-DhmRs relative to genomic features indicated a de-enrichment away from intergenic regions and enrichment at coding exons (Fig 1C indicated by *). When mapping the 5hmC-DhmRs across chromosomal locations, they were highly enriched towards the ends of chromosomes (Fig 1D). On the other hand, we failed to detect significant differences in 5mC levels between WT and MECP2- hiPSCs (data not shown), which may not be surprising considering that 5hmC is only thought to be a tiny fraction compared to

5mC. In addition, the effect of loss of MECP2 on 5hmC levels was strong enough to be observed by immunostaining in hiPSCs made from both patients (Fig 1E and F).

Loss of MECP2 affects the transcriptome of neurons

It has been suggested that loss of MECP2 only affects gene expression in neurons as opposed to the hPSCs and NPCs from which they were derived⁸. Coupled with the fact that 5hmC levels appear to be disturbed in MECP2 null hiPSCs, we sought to determine whether gene expression was affected in hiPSCs, NPCs or neurons in this patient derived *in vitro* model. We therefore proceeded with RNA seq (>120 million reads per sample) of homogenous hiPSC, NPC and interneuron cultures. With such sequencing depth, it was possible to analyze the RNA-seq reads for the known mutations present in the patients from which these lines were made (Fig S1). This analysis demonstrated that each line studied expressed strictly either the WT or mutant allele of MECP2, and that XCI status was unchanged even after extensive differentiation to neurons.

To optimize the search for molecular effects of loss of MECP2 in hiPSCs, NPCs or neurons, we generated more defined neuronal cultures by following the newly established 3i (three inhibitor) method to create populations of human interneuron progenitors (Fig S2A) and interneurons (Fig S2B)²¹. Interneurons are particularly relevant in the study of Rett Syndrome as interneuron-specific deletion of *Mecp2* in mice recapitulates many of the disease symptoms²²⁻²⁵. We validated the purity and quality of differentiation at each step by immunostaining for markers typical of particular cell types (SOX2, SOX1 and NESTIN as well as FOXG1 and NKX2.1 for NPCs; and Tuj1, MAP2 and GABA for

interneurons) in both WT and MUT cultures followed by quantification (not shown). We first assessed whether interneurons lacking MECP2 also showed diminished dendritic branching. In fact, in patient-derived interneurons made by 3i, defects in dendritic branching as measured by the number of endpoints were clearly observed (Fig 2A).

First, we quantified the expression level of MECP2 in WT cells across these three stages of development and found that the average RPKM was 3.1 for hiPSCs, 4.3 for NPCs, and 7.75 for interneuron cultures. This is consistent with consensus that MECP2 is enriched in neuronal cells, but also demonstrates that it could potentially be relevant to hiPSC and NPC physiology as well. However, high stringency analyses (FDR <0.05) of the RNA-seq data yielded very few gene expression changes due to loss of MECP2 in hiPSCs or NPCs derived from Rett patients (Fig 2B), consistent with Li et al⁸. On the other hand, interneuron cultures made from patient 982 showed many gene expression changes when comparing two individual WT and MUT clones (Fig 2B). Gene ontology analysis uncovered many neuronal physiology- related pathways were downregulated due to loss of MECP2 in neurons, while genes associated with extracellular remodeling and cell migration appeared to be induced (Fig 2C).

We then mapped the interneuron DEGs according to chromosomal location and found an interesting pattern whereby genes that many of the upregulated genes in the absence of MECP2 were enriched towards the ends of chromosomes (Fig 2D). Moreover, the same pattern emerged in analysis of low stringency DEGs (p value <0.05) from hiPSCs and NPCs lacking MECP2 (Fig S2C and S2D) for the upregulated genes. We did not observe

this pattern for the downregulated genes in hiPSCs, NPCs and neurons. These data suggested that MECP2 could play a role in gene regulation particularly at the ends of chromosomes, and was consistent with the pattern observed for hypomethylation of 5hmC in the absence of MECP2. Finally, there was also a small, but statistically significant overlap of 5hmC-DhMRs with DEGs in hiPSCs suggesting a link between the two (Fig 2E). These data suggest that loss of MECP2 leads to hypomethylation and transcriptional induction in subtelomeric regions of the chromosome.

We validated a number of the subtelomeric gene expression changes induced by loss of MECP2 by RT-PCR in independent preparations of hiPSCs, NPCs and Neurons (Fig 3A). Many of the subtelomeric genes upregulated are typically not expressed at all in hiPSCs, NPCs or neurons, thus the loss of MECP2 led to an aberrant expression pattern as opposed to a reinforcement or suppression of a typical pattern in these cell types. Furthermore, the subtelomeric genes that were induced in the absence of MECP2 were still typically present at less than 1 RPKM, clouding the potential physiological consequence of the induction of these genes.

To determine whether these effects were specific to the genetic background of the cells used or whether defects in reprogramming to the pluripotent state in the absence of MECP2 affected the downstream gene expression pattern, we silenced MECP2 in WT NPCs and assessed gene expression patterns in this context. Several different siRNA targeting oligos were assayed for their ability to silence MECP2 by RT-PCR, western blot, and immunostaining (Fig S3). Silencing of MECP2 in either WT-NPCs derived from other

pluripotent stem cells or WT-NPCs derived from fetal Medial Ganglionic Eminence (MGE) brain tissue²⁶, led to strong induction of expression of subtelomeric genes (Fig 3B and C).

Recently, it was discovered that a long non-coding RNA (lncRNA) is also transcribed from the subtelomeric domain into the telomeric sequence itself²⁷⁻³¹. This lncRNA is both known to be induced by telomere shortening and to potentially negatively regulate telomere length by competing for telomere priming within telomerase³⁰⁻³². TERRA transcripts are difficult to detect by RNA-seq because they contain mostly telomeric repeat sequences. This also makes it difficult to design PCR primers that are specific to a single TERRA transcript. However, we used established RT-PCR primers^{30,31} to show that, similar to many subtelomeric genes, several TERRAs were strongly induced in the absence of MECP2 in isogenic derivatives (hiPSCs, NPCs, interneurons) (Fig 3D). In addition TERRA was induced in WT-NPCs derived from pluripotent stem cells or from tissue (Fig 3D).

Loss of TET activity phenocopies loss of MECP2

To assess the possibility that MECP2 regulation of 5hmC levels is linked to the regulation of subtelomeric gene expression, we targeted TET enzymes by siRNA. TET enzymes convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), and their deletion or downregulation is known to severely diminish levels of 5hmC^{20,33}. According to RNA-seq, the three TET enzymes are expressed similarly in both WT and MUT patient neurons (Average RPKM: TET1, 4.1; TET2, 1.6; TET3, 3.6). RT-PCR demonstrated the ability to silence TET 1, 2, and 3 isoforms using a combination of siRNA oligos in neurons (Fig 3E).

Assaying for subtelomeric genes, including TERRA transcripts demonstrated that TET inhibition led to strong increases in not only subtelomeric coding genes, but also TERRA transcripts (Fig 3F), in a similar manner as in loss of MECP2, suggesting a link between 5hmC and subtelomeric gene expression. Together, these data confirm that loss of MECP2 can lead to induction of subtelomeric genes, including TERRA, and that this dysregulation could be due to an effect on 5-hydroxymethylation. Left unclear is how the loss of MECP2 leads to changes in 5hmC levels.

Loss of MECP2 leads to telomeric abnormalities

One of the most established functions for TERRAs is their ability to interfere with telomerase function by acting as a competitive inhibitor for telomere priming³⁴, leading to shortened telomeres. In addition, others have shown that decreased 5hmC in murine embryonic stem cells can also lead to telomere shortening³⁵, and, as shown here, loss of MECP2 led to decreased 5hmC in hiPSCs (Fig 1). Therefore, we attempted to determine the physiological consequence of TERRA induction in the absence of MECP2. qPCR for telomere length in fact showed that cells without MECP2 showed shorter telomeres, regardless of their stage of differentiation (Fig 4A) (a complete list of cell lines used for these analyses is provided in Supplemental Table 1). NPCs with transient siRNA knockdown of MECP2 also showed shorter telomeres, indicating that this effect was not simply due to defects during reprogramming or differential expansion of cell lines (Fig 4A). Quantitative fluorescence in situ hybridization (qFISH) was used to determine telomere length at the single cell level. qFISH demonstrated that NPCs without MECP2 showed significantly shorter telomeres relative to centromeric regions (Fig 4B). To further validate

these findings we performed southern blot with a telomere probe on NPCs with and without MECP2 and found telomere shortening in cells lacking MECP2 (Fig 4C). These data point towards telomere erosion in the absence of MECP2.

Telomere dysfunction is known to be present in some cancers, and also in cells driven to senescence due to telomere shortening, but this process has yet to be implicated in Rett syndrome etiology³⁶⁻³⁹. Telomere dysfunction is characterized by short telomeres, induction of PML, gammaH2aX, and p53⁴⁰. We assayed for evidence of telomere dysfunction by immunostaining for gamma-H2AX (Fig 5A) and PML (Fig 5B) in NPCs. WT NPCs with silencing of MECP2 by siRNA and neurons lacking MECP2 also showed clear induction of these marks (data not shown), consistent with telomere dysfunction induced by the absence of MECP2.

Induction of P53 and senescence pathways in the absence of MECP2

It is well established that shortening of telomeres puts significant stress on cells, which can lead to senescence or even apoptosis^{34,41-43}. As Rett Syndrome is caused by neuronal defects specifically, we determined how neurons lacking MECP2 respond to telomere shortening at the molecular and physiological level. Cells under stress due to telomere shortening are known to induce p53, which can then activate various response pathways downstream such as DNA repair, senescence, and apoptosis⁴⁴. Interestingly, p53 induction due to telomere shortening was previously shown to cause defects in dendritic branching^{43,45}, which is also the dominant phenotype in Rett Syndrome. Immunostaining for p53 in neurons with and without MECP2 showed a strong increase in

p53 protein in the absence of MECP2 (Fig 5C). p21, a transcriptional target gene of p53, was also induced in MECP2 null neurons at the protein level (Fig 5C). In addition, telomere shortening in NPCs due to overexpression of the Progerin allele, which is associated with accelerated aging also induced p53 expression (Fig S4E).

Because telomere shortening is known to also drive cellular senescence, we looked for signs of defective proliferation *in vitro*. While attempting to make clones of fibroblasts from patients with Rett syndrome, we repeatedly found that clones lacking MECP2 did not expand well after a passage (14 MECP2 null clones were created, none expanded), while clones expressing the WT allele expanded without problem (42 MECP2+ clones were created, and 4 out of 4 all expanded). To determine whether MECP2 null fibroblasts encounter senescence, we performed assays to detect endogenous beta-galactosidase, which is known to be a hallmark of this process⁴⁶. Indeed, MECP2 null fibroblasts showed strong activity in this senescence assay (Fig 5D).

We did not encounter such difficulties with clonal expansion once hiPSCs or hiPSC-derived NPCs were made from patients, presumably because during reprogramming, telomerase is strongly induced to restore telomere length at least beyond the critical threshold⁴⁷⁻⁵². In fact, our RNA-seq data showed that hiPSCs made from patients had very high expression of TERT, and NPCs still expressed moderate levels, while neurons did not express appreciable levels (average RPKM for TERT: hiPSC, 8.8; NPC, 1.6; neuron, 0.006). Importantly, the same endogenous galactosidase activity assay on interneurons showed a dramatic increase in senescence activity in neurons lacking

MECP2 (Fig 5E). On the other hand, similar assays on NPCs lacking MECP2 did not show any induction of senescence (data not shown). Together, these data indicate that loss of MECP2 leads to the generation of neurons that show evidence of telomere dysfunction.

Probing RNA-seq data, we also found that MECP2 null interneuron cultures showed a strong increase in a group of genes that are known to be induced by senescent cells, known as the Senescence Associate Secretory Program (SASP). Fig S4A shows that SASP genes were strongly induced in MECP2 mutant neurons, providing further evidence of a senescence phenotype. These senescence phenotypes are also intriguing in light of the transcriptional data suggesting an increase in aging- related genes by gene ontology analysis (Fig 2C). The only previous report linking MECP2 loss to senescence was performed by partial silencing of this protein in mesenchymal stem cells, but the results were consistent with those shown here for patient derived MECP2 null fibroblasts⁵³.

To demonstrate whether the induction of senescence and p53 observed here was due to telomere shortening as opposed to other molecular phenotypes due to loss of MECP2, we deliberately shortened telomeres in otherwise wildtype NPCs. We took advantage of the progerin allele of the Lamin-A gene. This truncated allele is similar to what is found in patients suffering from Progeria, a premature aging disorder typified by telomere shortening^{54,55}. Induction of the progerin allele by lentiviral infection of cDNA in WT NPCs showed a significant telomere shortening as expected (Fig S4B). In addition, induction of the progerin allele caused an increase in expression of the same subtelomeric genes

and TERRA transcripts that were induced by the loss of MECP2 (Fig S4C and S4D). This was presumably due to the Telomere Position Effect, whereby telomere shortening is known to lead to induction of subtelomeric gene expression^{56,57}. Importantly, progerin expression also led to a strong induction of p53 expression (Fig S4E), consistent with what was observed in MECP2 null neurons.

Blocking induction of P53 can rescue dendritic branching defects due to loss of MECP2

Previous evidence from a murine model of telomere shortening as a result of loss of telomerase complex (TERT) led to defects in dendritic branching, and this effect was strictly dependent on induction of p53⁴³. A more recent study also showed that experimentally aging the neural lineage with telomerase inhibition led to neurons with signs of aging, including reduced dendritic branching⁵⁸. Therefore, we posited that inhibition of P53 in MECP2 null neurons with shortened telomeres could potentially restore appropriate dendritic branching.

To determine whether blocking the action of P53 could improve dendritic branching in MECP2 null interneurons, we took advantage of Pifithrin- α , a potent inhibitor of P53 target gene activation⁵⁹. Treatment of MECP2 null interneurons with Pifithrin- α showed evidence of p53 inhibition as measured by RT-PCR for GADD45⁴⁴, a target gene important for DNA repair (Fig 5F). After 1-5 days of p53 inhibition by Pifithrin- α , MECP2 null interneurons appeared to adopt an improved neuronal morphology typified by increased physical distinction between the soma and neurites, longer, thinner neurites,

as well as increased dendritic branching as shown and quantified in Fig 5F. These data provide evidence that neurons with shortened telomeres due to loss of MECP2 respond by inducing P53 activity, which then inhibits the formation of complex neuronal processes. In summary, our *in vitro* model in human neurons suggests that loss of MECP2 leads to aberrant molecular regulation at the ends of chromosomes, leading to telomere shortening and a resulting induction of cell stress pathways such as p53 and senescence (Fig 5G).

Rett patient brains show evidence of telomeric dysfunction

To determine whether any of the phenotypes discovered in this *in vitro* model of Rett Syndrome have relevance to patients afflicted with the disease, we acquired tissue specimens from Rett Patients and aged matched controls. We first quantified the degree of chimerism of female Rett patient neurons due to skewing of X Chromosome inactivation to determine the relative ratio of neurons that express MECP2 versus those that did not. One of the Rett patient brains showed roughly 75% of its neurons lacking MECP2, while others appeared to have less than 25% MECP2 null neurons (Fig 6A). Southern blotting of the patient brain with 75% mutant neurons compared to an aged-matched control demonstrated that this Rett brain had shorter telomeres (Fig 6B). We then measured telomere length by PCR from genomic DNA isolated from small specimens of brain tissue from a group of Rett patients, and found that some aged matched Rett patient brains showed small decreases in average telomere length (Fig 6C), though not in every case (data not shown). Because of both the limited availability of Rett Patient brains, and the variable chimerism of WT and MUT neurons within these Rett

brains, perhaps it is not surprising that we were unable to detect trends across all brains analyzed using a method that cannot distinguish between WT and Mutant cells. This chimerism, coupled with the known variability of telomere length across even normal individuals and even across brain regions and cell types precludes an accurate assessment of general telomere length differences in Rett Brain until more samples become available for study.

On the other hand, we did find that the TERRA transcript was induced in nearly all Rett patient brains as measured by RT-PCR (Fig 6D), suggesting that all the Rett patients display dysregulation at the ends of their chromosomes. We did identify two Rett patient brains with a high proportion of MECP2 null neurons and subjected these to further investigation for signs of telomere dysfunction *in situ*. In patients 1815 and 5784, MECP2 null neurons showed a strong increase in both P53 and PML levels compared to adjacent neurons that expressed MECP2 (Fig 6E and F). This is consistent with the response to telomere shortening due to loss of MECP2 observed *in vitro*. These data are particularly intriguing in light of data showing that telomere shortening diminishes dendritic branching in various types of neurons and that this process can be dependent on p53 activity^{43,45}.

Discussion

Taken together, these data demonstrate that loss of MECP2 leads to telomere shortening, which in neurons results in clear signs of stress such as H2aX induction, p53/p21 induction, and initiation of a senescence program, all of which suggest that neurons in Rett Syndrome could be in suboptimal health, leading to neurophysiological defects such as dendritic arborization^{10,60}. Many of these phenotypes first observed in the *in vitro* model also appeared to be consistent with what could be observed in Rett patient brains, suggesting disease relevance for these findings.

It is curious that telomere defects have not been reported in previous models of Rett Syndrome. While one paper suggested that RNAi-mediated silencing of MECP2 could affect the telomeres of mesenchymal cells, decades of work on Rett Syndrome have not uncovered a role for MECP2 in relation to telomeres in a wide variety of models such as various transgenic mouse line, human patient post-mortem analyses, *in vitro* human models. Our study certainly benefited from analyses of multiple isogenically controlled cells from two patients and from the single cell analyses of patients with both WT and MUT neurons in the same area of the brain. This allowed for high confidence comparisons without having to correct for genetic background, or differences in tissue preparation. In addition, the study of telomeres in MECP2 mutant mice could be hampered by the simple fact that laboratory murine telomeres are on average 10x longer than human telomeres. Therefore, it is possible that telomere shortening in murine models does not proceed to such an extent by which one would expect induction of p53.

Patients with Rett Syndrome are typically characterized by a normal development at birth and subsequent failure to thrive leading to microcephaly and intellectual disability that develops with age. As a result, Rett Syndrome is thought to be caused by experience-dependent loss of neuronal function, which would correlate with data suggesting that MECP2 regulates activity dependent gene expression⁶⁰⁻⁶². The microcephaly has been proposed to be a function of decreased nuclear size and dendritic arborization of affected neurons^{10,60}. Could the telomere shortening induced senescence described here underlie patient phenotypes? Several studies have looked at the effects of telomere shortening specifically in the neural lineage and found consistently that shortened telomeres leads to upregulation of p53 and decreased dendritic arborization^{43,45,63}, a phenotype widely described to afflict MECP2 null neurons *in vitro* and *in vivo* (see chapter 2).

These results presented here raise the question of whether telomere defects could be common to the etiologies of other ID syndromes. The phenotypes described here show a striking similarity to those observed in hiPSCs and neural derivatives made from patients with ICF Syndrome⁶⁴⁻⁶⁶. Two independent studies showed that ICF patient-derived hiPSCs displayed subtelomeric hypomethylation, induction of subtelomeric gene expression, TERRA induction and telomere shortening that was coupled to senescence of somatic derivatives such as fibroblasts. ICF Syndrome only partially overlaps with Rett Syndrome in terms of patient phenotypes, but is caused by mutations in DNMT3B, a *de novo* DNA methyltransferase⁶⁷. These findings together are highly relevant as DNMT3B is a key *de novo* methyl transferase to create methylated DNA (5mC), which is the substrate for Tet oxygenases to create 5-hydroxymethylated DNA (5hmC). Recently,

another study showed that deletion of Tet enzymes, which are critical to generate the 5hmC mark, led to shortened telomeres^{35,68}. Together, these studies demonstrate that DNA hydroxymethylation is important in the regulation of telomere length, and our data suggest that MECP2 is potentially an important mediator of this effect.

ATRX-related syndrome shares many phenotypic features with Rett Syndrome (ID, seizures, and microcephaly), and the causative gene, ATRX, is known associate with MECP2 both genetically as well as biochemically⁶⁹⁻⁷⁴. In murine models of loss of ATRX, telomere shortening and reduced lifespan are observed⁷⁵. The fact that the causative mutations of ICF, Rett and ATRX syndromes are in genes whose products are thought to interact in the regulation of DNA methylation and all possess telomeric defects suggests that the similarities of patient phenotypes could be the result of neuronal response to telomere shortening. This molecular overlap could even form the basis of novel therapeutic strategies that either reverse telomere shortening or block the response of the cell to telomere defects with agents such as Pifithrin, as shown in Fig 5F.

Considering the phenotypes of ICF derived cells, and those of Tet-deleted cells, it seems reasonable to suggest that telomere deficiency could be related to intellectual disability. In addition, subtelomeric and telomeric dysfunction has been implicated in up to 10% of all intellectual disability syndromes⁷⁶⁻⁷⁸. Another ID syndrome, Hoyerhaal-Hreidarsson, is caused by mutations in RTEL1 (regulator of telomere elongation helicase 1), a factor that interacts with shelterin complex and is critical for telomere elongation⁷⁶. These patients are characterized by low birth weight, microcephaly and immunological dysfunction.

Therefore, mutations that specifically result in telomere shortening lead to disease phenotypes similar to those found in patients with loss of MECP2. As a result, we cannot exclude the possibility that telomere shortening during *in utero* development generates neurons that are less well equipped to deal with post-natal stimulation. Furthermore, our analysis of the single male Rett brain specimen available to us suggested that brains completely lacking MECP2 also had shorter telomeres.

Another possible interpretation of these data is that instead of a failure to mature, Rett Syndrome neurons instead show aspects of premature aging. The fact that MECP2 null neurons have shorter telomeres (Fig 4), show induction of aging related genes including p53 (Fig 2 and 5), and show senescence (Fig 5) are all consistent with this idea⁷⁹. In addition, the fact that WT-NPCs and neurons transduced with the Progerin allele⁸⁰, which is known to cause premature aging, show similar phenotypes as to neurons lacking MECP2 is also consistent with this idea. On the other hand, while Rett patients suffer from a post-natal cognitive decline, and long term survivors show phenotypes associated with Parkinson's disease⁸¹, the typical phenotypes presented in young female patients are not consistent with premature aging. Whether the physiological response to loss of MECP2 is truly akin to premature aging or whether patients suffer from the effects telomere dysfunction that is unrelated to aging is worthy of continued investigation.

Regardless, it is tempting to speculate that treatments that could relieve telomere dysfunction or abrogate the p53 mediated stress response could potentially ameliorate patient outcomes. Pifithrin- α has already been shown to be an effective treatment to

restore neuronal function in murine models of injury or stroke⁸²⁻⁸⁴. Significant future effort will be devoted to determining both whether telomere dysfunction is a common trigger for ID Syndromes, and whether telomere restoration could potentially help patients.

Figures

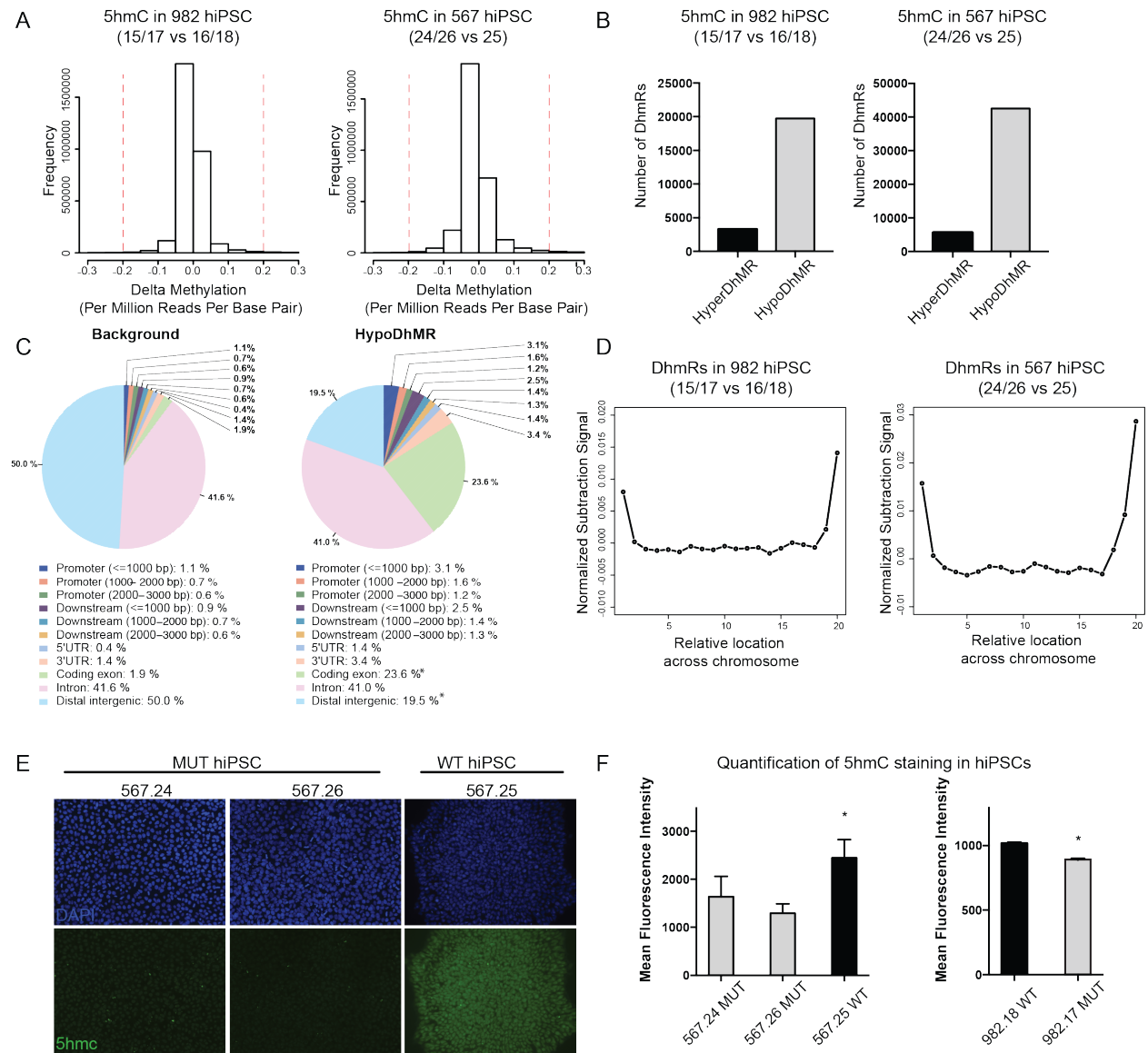
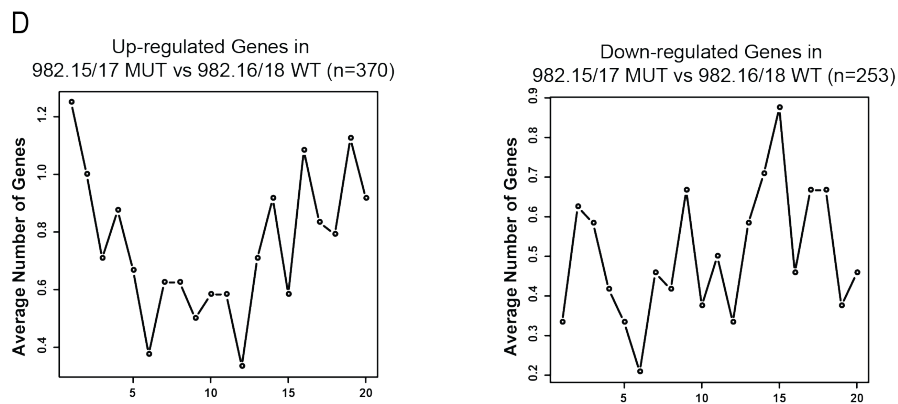
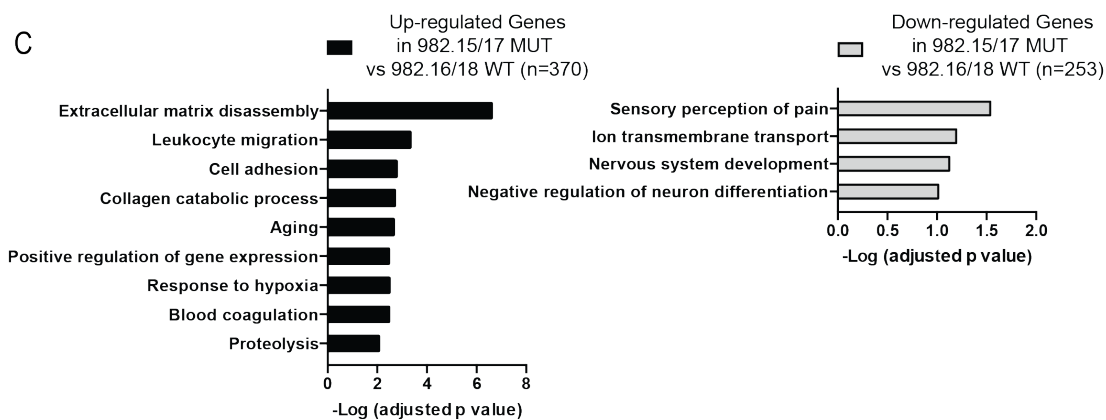
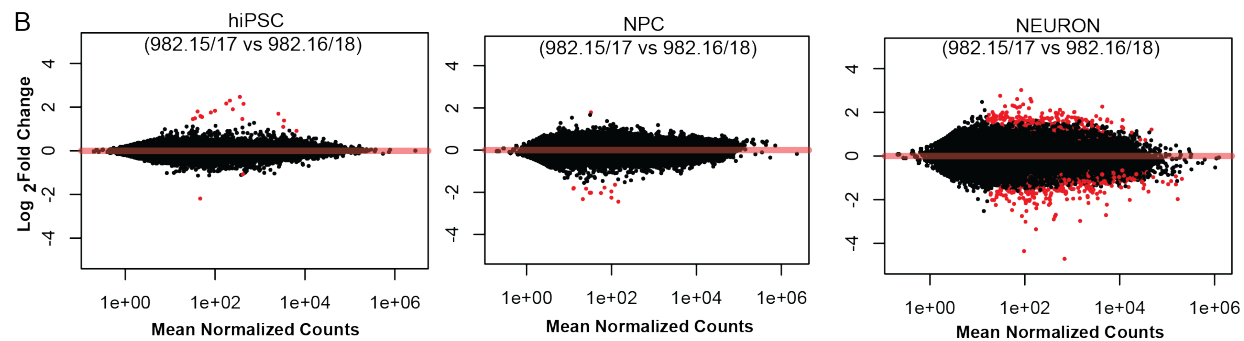
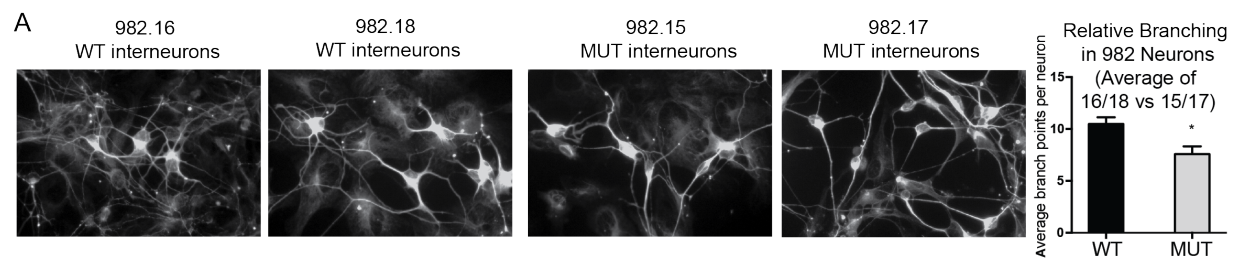


Figure 3-1. Hypomethylation of 5-hydroxymethylcytosine in MECP2 null cells

A, The overall delta methylation signal distribution is shown. The cutoff was made based on the difference of 0.2 per million reads per base pair. B, Number of differential 5hmC regions (DhmRs) are presented as either gain of 5hmC (hypermethylated) or loss of 5hmC (hypomethylated) in each patient line, comparing MECP+ clones to MECP2- clones. Differential hydroxymethylation pattern between clones from 982 and 567 shows

the overall delta-methylation as hypomethylation. C, Localization of DhmrRs within various genomic features relative to the portion of those features in the genome. The highest concentration of DhmrRs was found in coding exons (light green). D, Mapping DhmrRs across metachromosomes representing the relative location across all chromosomes shows an increase in DhmrRs towards the ends of chromosomes. The y-axis represents the differences of normalized methylation signal (piled-up signal per million mapped reads) between wild-type and mutant. E, Immunostaining for 5hmC in hiPSC clones from patients 982 and 567 indicated that levels of this DNA methylation mark are considerably lower in MECP2 null hiPSC clones. F, 5hmC staining was quantified in hiPSCs derived from both patient 567 and 982. *p value < 0.05 according to student's t test. Bar graphs represent mean +/- SEM.



E

Number of DEG (hiPSCs)	Overlap with Hyper DhMR	Overlap with Hypo DhMR
20	5 (1)	19 (6)

Figure 3-2. Loss of MECP2 is associated with differential gene expression particularly in neurons

A, Immunostaining neurons generated from patient 982 for TuJ1, a neuronal-specific marker. Right, quantification of dendritic complexity by counting endpoints shows a significant difference between neurons with and without MECP2 made from patient 982. B, Volcano plots of differentially expressed genes (DEGs) in hiPSCs, NPCs and Neurons shows that loss of MECP2 has a profound effect on gene expression in neurons. C, Gene ontological analysis of DEGs increased versus decreased in MECP2 null neurons. D, DEGs were mapped to a metachromosome to determine their relative location across chromosomes. In general, upregulated DEGs were enriched towards the ends of chromosomes, while downregulated DEGs showed no clear pattern of location. E, DEG and Dhmr are statistically significantly overlapped in hiPSCs. This analysis was performed by randomly select the same number of genes with iPSC FDR DEG from the hg19 genes, then calculating the overlapping with hmC DMRs in a permutation test performed 5,000 times. The permuted number of overlapping genes is shown in the parentheses.

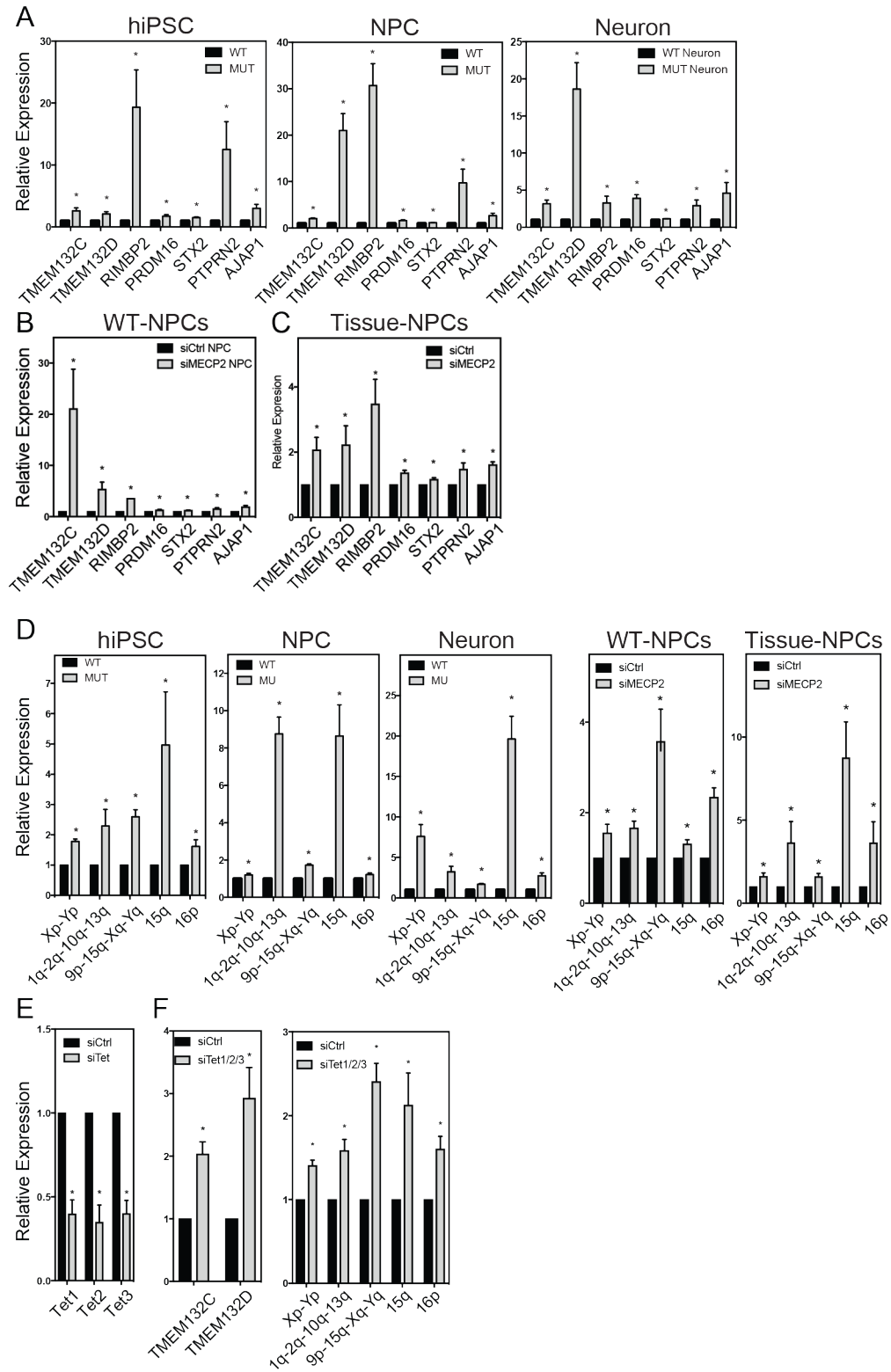


Figure 3-3. Loss of MECP2 leads to induction of subtelomeric genes including TERRA, a long non-coding RNA

A, RT-PCR for subtelomeric genes in hiPSCs, NPCs and neurons derived from patients. B, RT-PCR for subtelomeric genes in WT NPCs with silencing of MECP2 by siRNA. C, RT-PCR for subtelomeric genes in WT brain tissue derived NPCs with silencing of MECP2 by siRNA. D, RT-PCR with the same samples described in A, B and C for TERRA transcripts. E, Silencing of TET expression by siRNA was assessed by RT-PCR. F, Knockdown of TET followed by RT-PCR for TERRA transcripts and subtelomeric genes also showed that loss of 5hmC is associated with induction of subtelomeric gene expression. In this figure, all data presented are the resulting relative fold change differences found in at least three biologically independent experiments. In addition, student's t-test was performed across all three or more experiments, and those with a p-value < 0.05 are indicated with an asterisk. Bar graphs represent mean +/- SEM. The identity of cells used in each replicate experiment are described in Supplemental Table 1.

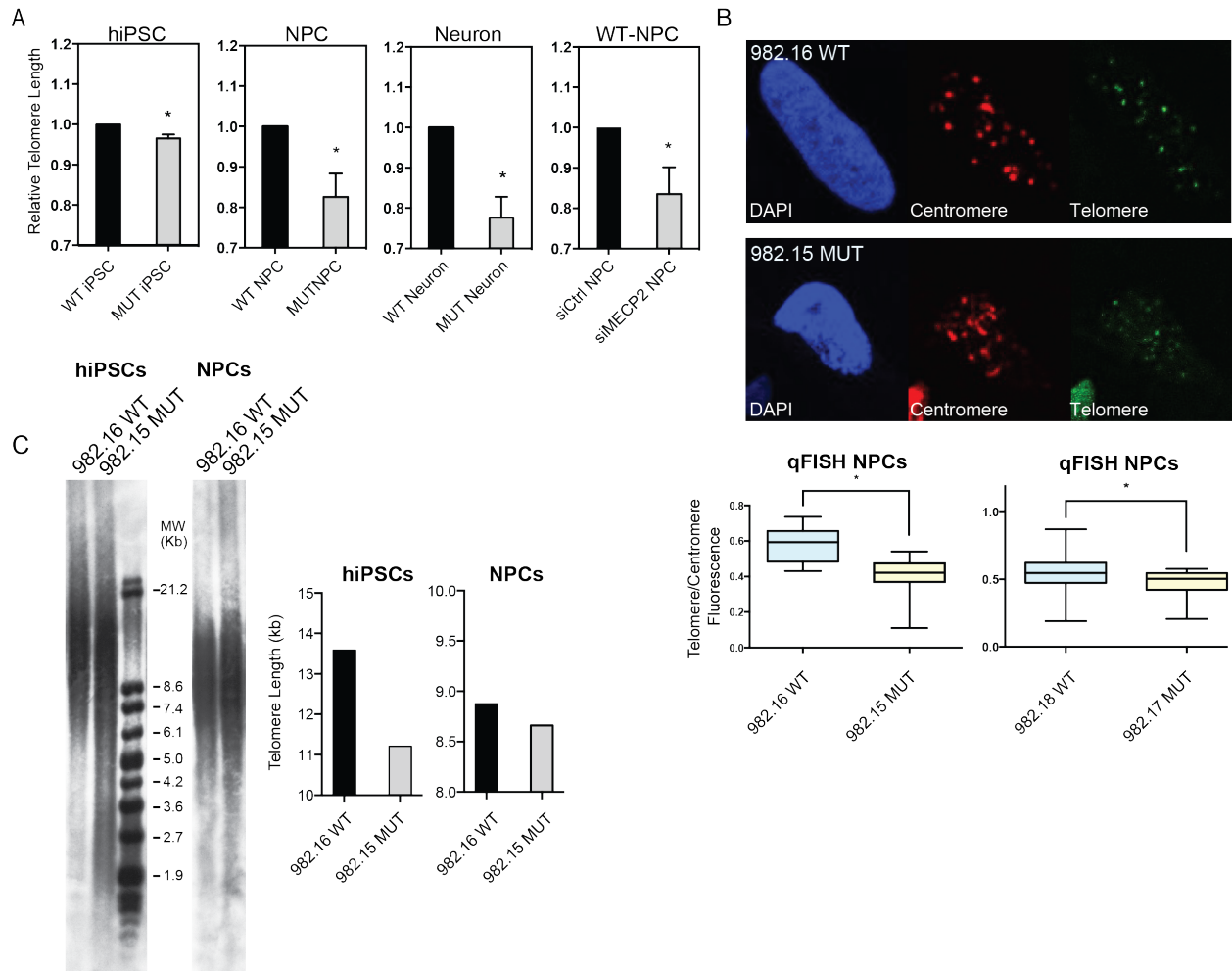


Figure 3-4. Loss of MECP2 is associated with telomere shortening

A, Quantitative PCR for telomere length based on a ratio of telomere product versus an autosomal locus (T/S ratio) showed that loss of MECP2 in patients or by siRNA for MECP2 is associated with shorter telomeres in hiPSCs, NPCs and neurons. The data presented are the result of at least three biologically independent experiments, and asterisks indicated p-value < 0.05 according to student's t test. Bar graphs represent mean +/- SEM. A complete sample list across all experiments used is provided in Supplemental Table 1. B, As an independent method, quantitative FISH was performed for telomere length as a function of centromere size. Bottom, quantification of telomere length in NPCs in two separate experiments from patient 982. C, Southern blotting with

genomic DNA and a telomere specific probe showed telomere shortening in the absence of MECP2 in hiPSCs and NPCs derived from patient 982.

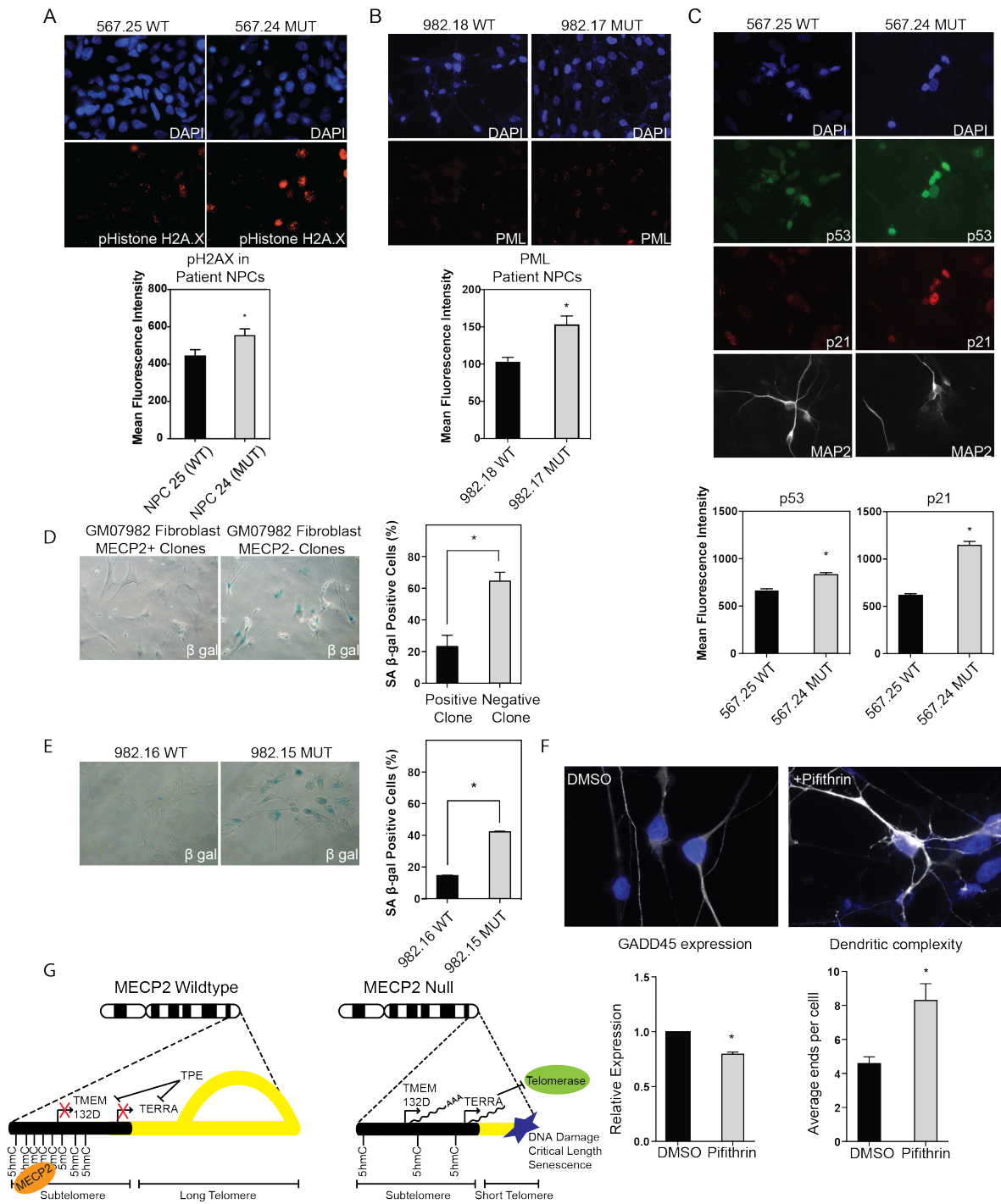


Figure 3-5. Physiological consequences to telomere shortening in the absence of MECP2

Immunostaining for H2aX and PML can identify cells with telomere dysfunction. A, Immunostaining NPCs in the absence of MECP2 showed a strong increase in H2aX, which was quantified as a function of SOX2 immunostaining in 567 derived NPCs. B, Immunostaining NPCs in the absence of MECP2 showed a strong increase in PML, which was quantified as a function of SOX2 immunostaining in 982 derived NPCs. C, Immunostaining for p53 and p21, a target of p53, showed an increase of these stress markers in MECP2 null neurons derived from patient 567. D, Cells undergoing senescence show upregulation of endogenous b-galactosidase activity. Clones of fibroblasts lacking MECP2 showed strong b-gal activity, while those of WT fibroblasts did not. E, The senescence assay applied to neuronal cultures showed a strong increase in the absence of MECP2. F, Treatment of MECP2-null neurons with DMSO or Pifithrin, followed by immunostaining with antibody for TuJ1 shows a change in dendritic branching and morphology following treatment with Pifithrin. Bottom left, RT-PCR for GADD45, a p53 target gene, showed that Pifithrin reduced p53 activity. Bottom right, Quantification of branching phenotype across three independent experiments showed a strong increase in branching as measured by the number of endpoints. G, Schematic to depict molecular events known to regulate the ends of chromosomes. Shown on the right is the result of loss of MECP2, which according to immunostaining and senescence activity assays, led to neuronal stress and TIF. In this figure, all data resulted from at least three independent experiments. *p value < 0.05 according to student's t test. Bar graphs represent mean +/- SEM.

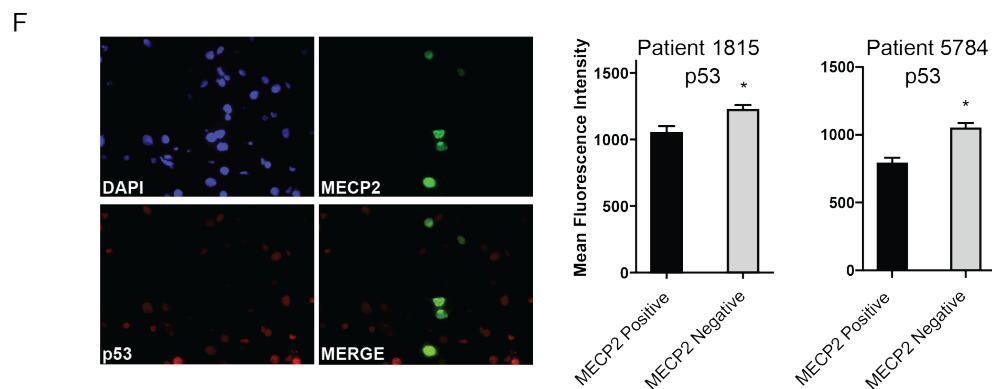
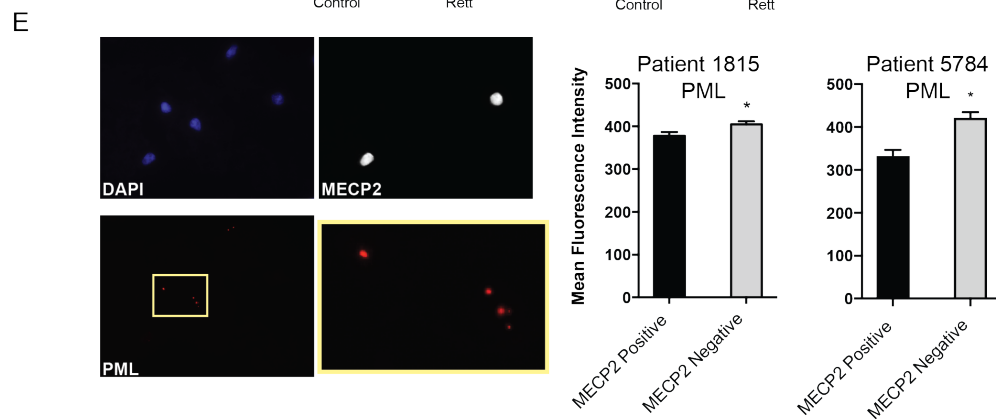
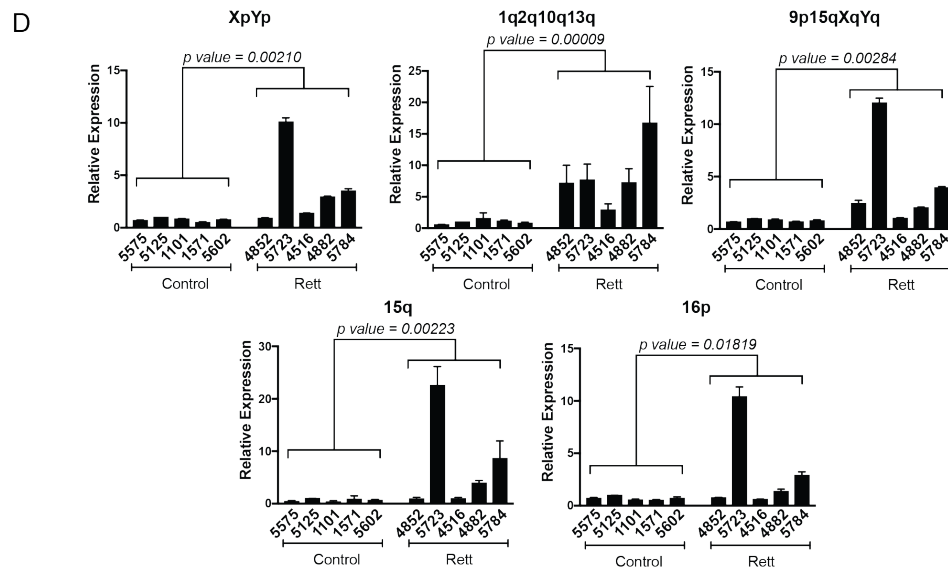
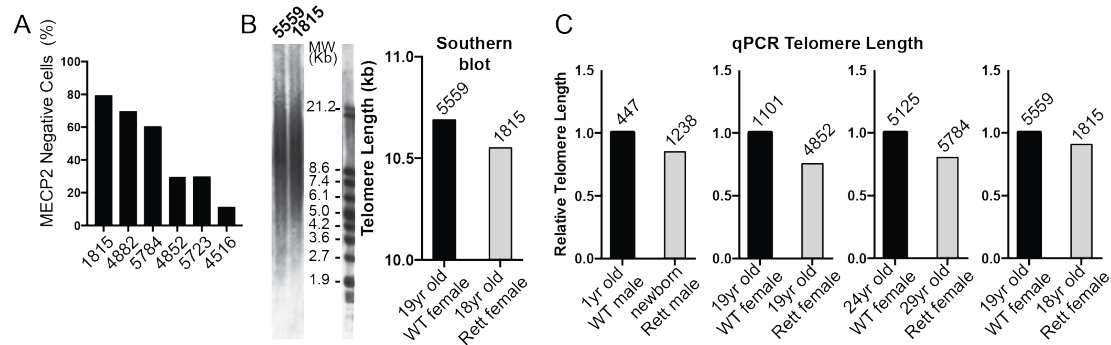
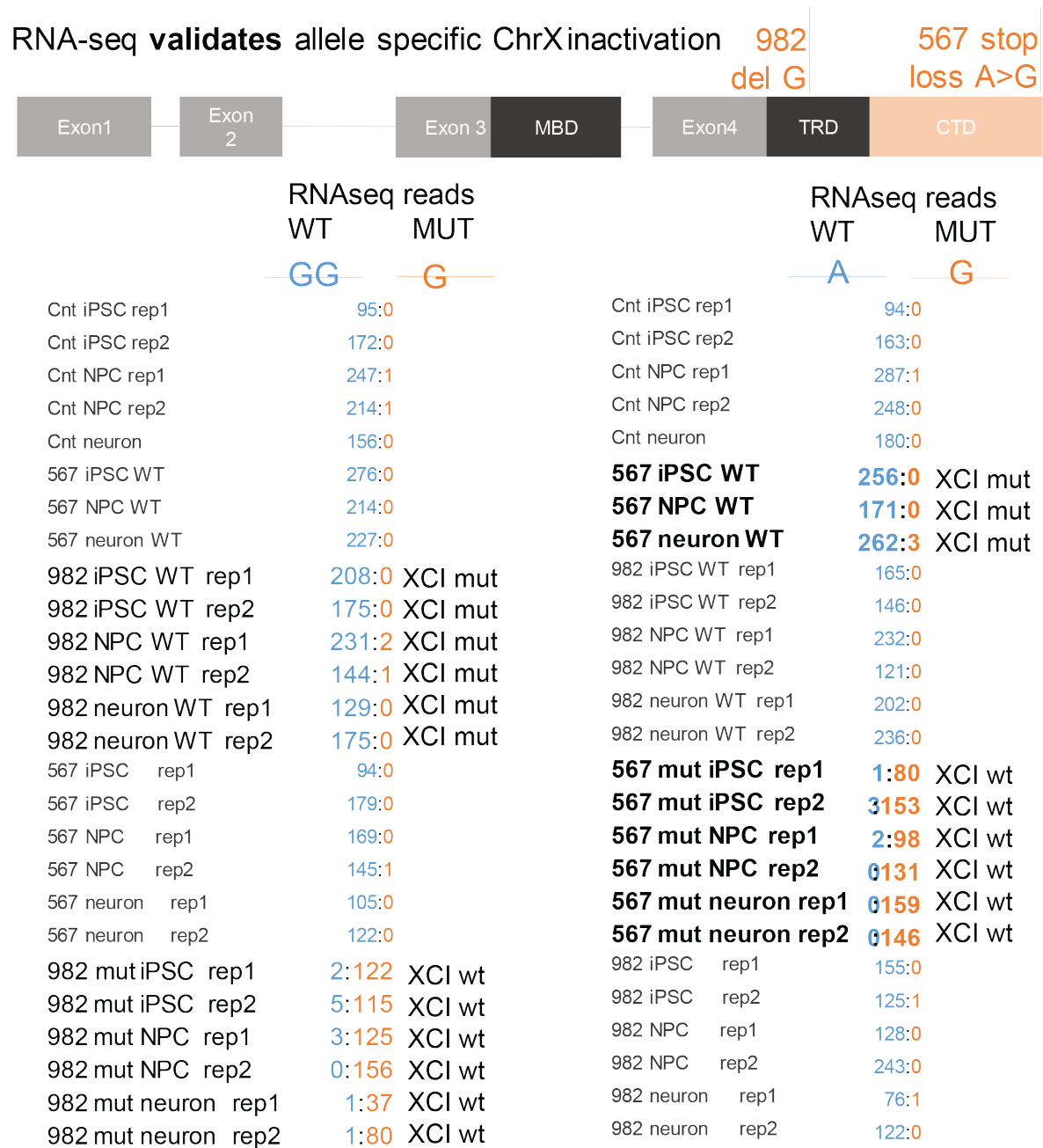


Figure 3-6. Rett patient brains show telomere shortening and induction of p53

A, Female Rett patient brains show variable XCI skewing in neurons as judged by immunostaining for MECP2, and quantified as a function of DAPI and MAP2 staining. B, Southern blotting of genomic DNA with a telomere specific probe shows the average telomere length in both control (5559) and Rett (1815) brain compared to ladder (right). C, qPCR from genomic DNA of aged matched Rett patient brains and control brains. D, As measured by RT-PCR, Rett patient brains show an increase in TERRA transcripts compared to aged-match controls. N $\geq$ 3 independent experiments. Bar graphs represent mean $\pm$ SEM. E, Extended characterization of patient 1815 and 5784 showed increased expression of PML specifically in MECP2 null neurons in each of these two patient brains. Yellow inset is a magnification of box showing high magnification of PML staining specifically in MECP2 null neurons. Right panel shows quantification of PML signal in Rett patient brain, comparing the signal in MECP2+ versus MECP2- neurons. F, Immunostaining Rett brain for MECP2 and p53 shows higher levels of p53 specifically in MECP2- neurons (quantified on the right).

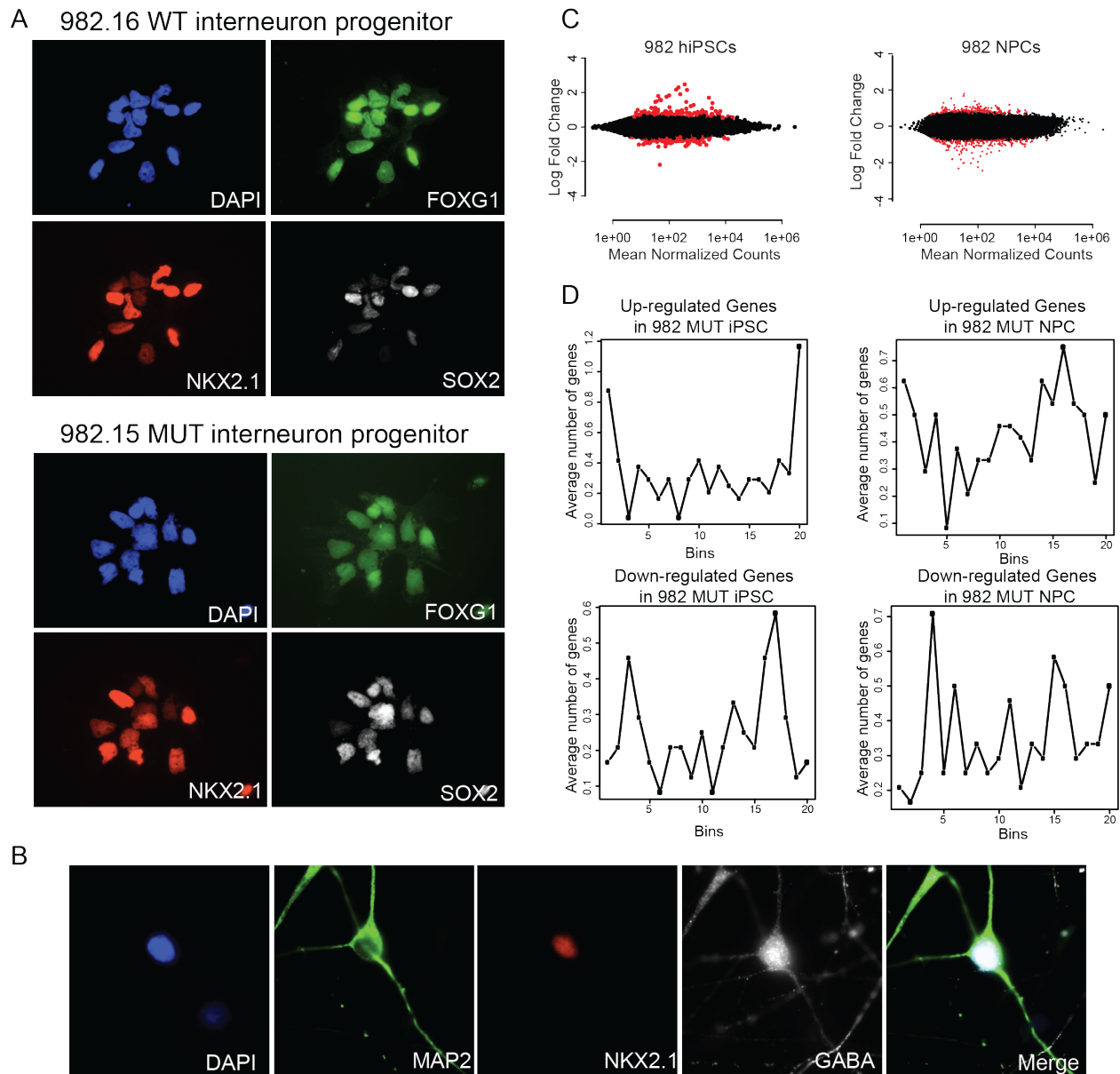
Supplementary Figures



Supplementary Figure 3-1. RNA-seq analysis to determine the relative ratio of WT versus MUT transcripts of MECP2 in Rett patient derived lines

Detection of WT and MUT transcripts from each of the lines indicated demonstrated a clear bias towards individual alleles in each patient derived line. This analysis indicates

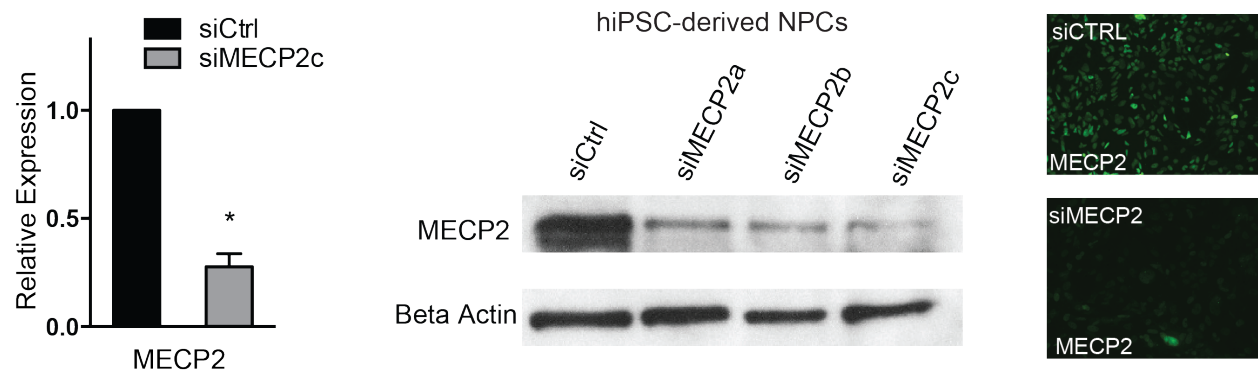
XCI status for each allele, and demonstrates that XCI status is unchanged, even after differentiation to neurons.



Supplementary Figure 3-2. Low stringency analysis of DEG in hiPSCs and NPCs

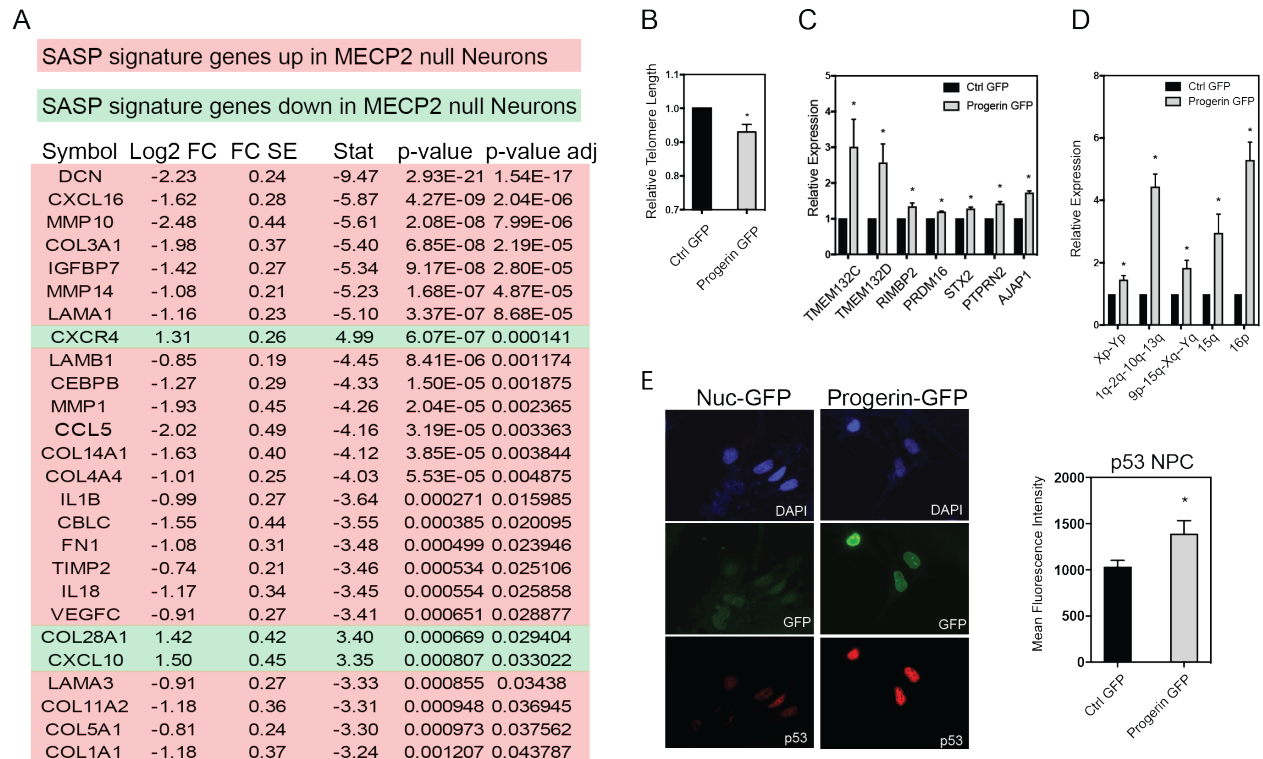
A, Immunofluorescence of interneuron progenitors from WT (top) or MECP2null (bottom) clones. B, Immunofluorescence of interneurons generated from a MECP2null hiPSC clone generated by 3i protocol. C, Volcano plots of lower stringency DEGs in

hiPSCs and NPCs between MECP2+ versus MECP2- clones (p-value < 0.05). D, Mapping of low stringency DEGs in hiPSCs and NPCs across metachromosome to measure enrichment of DEG location.



Supplementary Figure 3-3. Silencing MECP2 by siRNA

MECP2 was downregulated by RNA interference, quantified by RT-PCR (left), for protein by western blot (middle), and as demonstrated by immunostaining for MECP2 (right).



Supplementary Figure 3-4. Transduction of Progerin leads to phenotypes similar to loss of MECP2

A, Cells undergoing senescence are known to induce and secrete a group of genes called SASP. RNA-seq data from neurons were mined for SASP genes, and shown are those SASP genes that were differentially expressed between patient derived neurons with and without MECP2. B, qPCR for telomere length in WT NPCs showed that Progerin infected cells have on average shorter telomeres. N=3 independent experiments. *p value < 0.05 according to student's t test. Bar graphs represent mean +/- SEM. C, RT-PCR from progerin infected NPCs showed an induction of various subtelomeric genes. N ≥3 independent experiments. *p value<0.05 according to student's t test. Bar graphs represent mean +/- SEM. D, RT-PCR for TERRA transcripts following progerin transduction. N ≥3 independent experiments. *p value<0.05 according to student's t test. Bar graphs represent mean +/- SEM. E, Immunostaining for p53 following progerin

expression. Quantification of p53 in infected cells (either Nuclear-GFP or Progerin-GFP) is shown on the right. *p value<0.05 according to student's t test. Bar graphs represent mean +/- SEM.

Supplementary Tables

TERRA			Subtelomeric genes			Telomere	
hiPSCs		NPCs	Neurons	hiPSCs	NPCs	Neurons	
N gene	Sample	N gene	Sample	N gene	Sample	N gene	Sample
3Xp-Yp	24/25	4Xp-Yp	17/18	3Xp-Yp	24/25	TMEM132D	15/16
	25/26		17/18		26/25	3Exon	15/16
	25/26		17/18		25/26		15/16
			17/18		25/26		15/16
			17/18		25/26		15/16
			17/18		25/26		15/16
51q_2q_10q_13q	15/16	1q_2q_10q_13q	15/16	TMEM132C	17/18	TMEM132C	17/18
	15/16	313q	26/25	Exon	25/26	3Exon	24/25
	24/25		17/18		25/26		17/18
	25/26		17/18	4RIMBP2	15/16	3RIMBP2	25/26
	15/16	9p_15q_Xq_	17/18		17/18	4RIMBP2	L17/L18
		3Yq	26/25		24/25		P15/P16
			L15/L16	4PTPRN2	17/18		24/25
39p_15q_Xq_Yq	15/16				25/26	5PTPRN2	24/25
	15/16				17/18		24/25
	24/25	315q	26/25		17/18		24/25
	15/16		24/25		17/18		24/25
615q	15/16		26/25	4AJAP1	17/18		24/26
	15/16		L17/18		15/16		17/18
	17/18	316p	17/18		17/18	4STX2	L15/L16
	24/25		24/25		17/18		P15/P16
	15/16			3STX2	15/16		3mt
					17/18		P15/P16
316p	17/18				15/16	4PRDM16	3mt
	15/16			3PRDM16	17/18		P17/P18
	24/25				15/16		3mt
					17/18		24/25
						4STX2	L15/16
							L17/L18
							P15/P16
							P17/P18
						3PRDM16	L17/L18
							P15/P16
							P17/P18

Supplementary Table 1. A list of samples used for RT-PCR and telomere qPCR

Provided is a list of all cell types analyzed across the RT-PCR and Telomere qPCR experiments performed in this manuscript.

Gene	Forward (3' → 5')	Reverse (3' → 5')
B-Actin	GATCATTGCTCCTCCTGAGC	AAAGCCATGCCAATCTCATC
MECP2	GCTCTGCTGGGAAGTATGATG	ATGTGTGCGCCTACCTTTTCG
TMEM132C	CATGAAGCTGGAGGAACCTCAC	GTCTTCTCTGCCAGGATGGAGT
TMEM132D	CAGACTGGCAAGTGGACATCAC	GGATGGTGTCTGACAGAGGAGA
RIMBP	AGGACGCCTATGACTCTCCAGA	CTCTGTGTGGTACTCGTCTCCA
PTPRN2	TTCTCGGACCAGCAGTGACCTT	TCAGTCCAGAGGTTTCCTCCAG
AJAP1	GTTAGCACAACGGAGCCTTCCA	TGATGAGGGAGACGGTGATGGT
PRDM16	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG
STX2	AGACCAGCATCCGAGAGTTGCA	GCGTGTTCTACATAGTCTGTGGC
TTC34	ACTGCACGCATGTTTCCTTCT	TGGCCTTCAGTCTCTTCAGG
9p15qXqYq	TTCCGCACTGAACCGCTCTAA	GCAGCCATGAATAATCAAGGT
1q2q10q13q	GAATCCTGCGCACCGAGAT	CTGCACTTGAACCCTGCAATAC
XpYp	GCAAAGAGTGAAAGAACGAAGCTT	CCCTCTGAAAGTGGACCAATCA
15q	CAGCGAGATTCTCCCAAGCTAAG	AACCCTAACCACATGAGCAACG
16p	TGTGTTTCAACGCTGCAACTG	AGTTAGAACGGTTCAGTGTG
GADD45a	CGTTTTGCTGCGAGAACGAC	GAACCCATTGATCCATGTAG

Supplemental Table 2. A list of primers

Provided is a list of all the primers used in this study.

Acknowledgements

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Materials and Methods

Differentiation in vitro and analysis

For directed differentiation of interneurons, iPSCs were grown on plates coated with matrigel (Corning) until 80% confluency with mTeSR (Stem Cell Technologies). Cells were then treated with DMEM/F12 (GIBCO) containing NEAA (GIBCO), GlutaMAX (GIBCO), bovine serum albumin (Sigma-Aldrich), β -mercaptoethanol (Sigma-Aldrich), N2 (GIBCO), B27 (GIBCO), SB431542 (10uM; Cayman Chemical), LDN-193189 (100nM; Cayman Chemical) and XAV939 (2uM; Cayman Chemical) later transitioning to the media containing sonic hedgehog (20ng/mL; R&D) and purmorphamine (1uM; Cayman Chemical) as previously described (Maroof et al., 2013). Cells were further differentiated into interneurons with neurobasal medium (GIBCO) containing N2 (GIBCO), B27 (GIBCO), ascorbic acid (Sigma-Aldrich), GlutaMAX (GIBCO), bovine serum albumin (Sigma-Aldrich), β -mercaptoethanol (Sigma-Aldrich), neurotrophin-3 (10ng/mL; R&D), brain-derived neurotrophic factor (10ng/mL; R&D), and glial cell-derived neurotrophic factor (10ng/mL; R&D).

Western blot

Cells were lysed on ice with RIPA buffer (Pierce) that contains Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). The total protein concentration was measured using BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's protocol. The lysates containing the equal amount of total protein were mixed with NuPAGE sample buffer (Invitrogen) with 5% mercaptoethanol and denatured at 95 °C for 10 min. Supernatant

was electrophoresed onto NuPAGE 4-12% Bis-Tris Protein Gels (Invitrogen) using MOPS running buffer (Invitrogen). Gels were then electroblotted using semi-dry transfer apparatus onto a membrane. The membrane was blocked with 5% non-fat milk for 1 hr and incubated overnight with primary antibodies at 4°C. The next day the membrane was washed and incubated with HRP-conjugated secondary antibodies for 1 hr at room temperature. The membrane was then incubated with ECL Western Blotting Substrate and developed.

Immunofluorescence and image quantification

Cells on coverslips were washed with PBS, fixed in 4% paraformaldehyde for 15 min at room temperature, blocked for 1 hr at room temperature with 10% serum and 0.1% Triton-X-100, then incubated overnight at 4 °C with primary antibodies. Frozen tissue sections were thawed to room temperature, fixed in 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.2% Triton-X-100 for 15 min at room temperature and blocked with 10% serum at 4 °C overnight, followed by incubation with primary antibodies at 4 °C for 16-24 hr. Following primary antibody incubation, the coverslips were incubated with Alexa Fluor (Invitrogen) or Jackson immunoresearch secondary antibodies at room temperature for 1 hr. Cells were counterstained with DAPI and mounted in Prolong Gold (ThermoFisher). Antibodies used include the following: mouse anti-OCT3/4 (1:100, Santa Cruz Biotechnology Inc.), rabbit anti-SOX2 (1:300, Cell Signaling Technology), rabbit anti-Nanog (1:100, Cell Signaling Technology), mouse anti-Tra-1-81 (1:250, Chemicon), mouse anti-NESTIN (1:1000, Neuromics), chicken anti-MAP2 (1:2000, Biolegend), chicken anti-GFAP (1:2000, Abcam), rabbit anti-Tubulin β 3 (1:500, Covance), mouse

anti-p53 (1:500, Cell Signaling), rabbit anti-p21 (1:250, Santa Cruz), mouse anti-PML (1:100, Santa Cruz), mouse anti-phospho-Histone H2A.X (1:2000, EMD Millipore), rabbit anti-5hmc (1:100, Active Motif), rabbit anti MECP2 (1:1000, Diagenode), rabbit anti Foxg1 (1:1000, Abcam), and mouse anti NKX2.1 (1:300, Novocastra). Secondary antibodies conjugated with Alexa 488, 568, 594, 647 (1:500, Life Technologies) were used. Imaging was performed on Zeiss Axio Imager A1 or Zeiss LSM780 confocal microscope using a 40X or 63X objective on randomly selected cells. Mean intensity or a number of foci were quantified using ImageJ (<http://rsb.info.nih.gov/ij/>). At least 100 cells per condition were used for each independent experiment.

RT-qPCR

RNA from cultured cells was collected using the RNeasy Mini Kit from Qiagen according to the manufacturer's instructions. The concentration and purity of RNA were measured using nanodrop spectrophotometers (Thermo Scientific). RNA with an A260/A280 ratio in between 1.8 and 2.0 as well as an A260/A230 ratio in between 2.0 and 2.2 was used. RNA was then reverse transcribed using the Super Script III First-Strand cDNA Synthesis kit with Random Hexamers (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was performed using SYBR Green master mix (Roche). Primers were used at a final concentration of 1 μ M. Reactions were performed in duplicate and duplicate CT values were averaged and then used for standard $\Delta\Delta$ CT analysis. Expression levels were normalized to beta actin. See Supplementary Table 2 for qPCR primer sequences.

Data collection and statistical analysis

All the experimental data (RT-qPCR, qPCR assay for telomere length, immunostaining, β -Galactosidase Senescence Assay) were presented as mean $\pm$ SEM based on at least three biological replicates from independent experiments using the cell lines indicated in Supplementary Table 1. Student's t tests were applied to data with two groups. ANOVA analyses were used for comparisons of data with greater than two groups. A p value < 0.05 was considered as statistically significant.

siRNA gene silencing

All knockdown experiments were performed using trilencer siRNAs (from OriGene Technologies) and RNAimax (ThermoFisher) in Opti-MEM media (ThermoFisher). Trilencers were used at a concentration of 20 nM. Transfection media was prepared and then 500,000 cells were plated on top of the transfection media in 6-well plates. The medium was changed to normal NPC media the next day and cells were collected for analysis at the time points indicated.

β -Galactosidase Senescence Assay

β -Galactosidase Senescence Assay was performed using the Senescence β -Galactosidase Staining Kit from Cell Signaling according to manufacturer's instructions. Briefly, the cells were fixed on coverslips, incubated with X-gal overnight at 37°C, then mounted on glass slides and imaged using a brightfield microscope. The number of blue cells and number of total cells were quantified using the Cell Counter plugin in ImageJ.

Quantitative fluorescence in situ hybridization

Cells were fixed with 4% paraformaldehyde for 15 min at RT and permeabilized with 0.5% Triton X for 10 min at RT. After dehydration series of 80, 95, and 100% cold ethanol, cells were then treated with RNase (100ug/mL in 2xSSC) for 30 min at 37°C. After washing and another dehydration series, cells were denatured at 85°C for 15 min in the hybridization mix (70% DI formamide, 10 mM Tris-HCl, pH 7.5, 2xSSC, 0.1ug/mL salmon sperm DNA) containing PNA probes (TelC-FITC and Cent-Cy3, Panagene) at 1ug/mL and then incubated for 2 hr at RT in dark. After hybridization, cells were washed 3X for 5 min in 2xSSC/50% DI formamide, in 2xSSX with 0.1 Tween20, and in 1xSSC. Cells were then counterstained with DAPI and mounted with ProLong Gold (ThermoFisher).

Southern blot analysis of terminal restriction fragments (TRF)

Total genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instruction. Fresh genomic DNA with high purity (an A260/A280 ratio of 1.8) was used for experiments. The integrity of genomic DNA was determined by gel electrophoresis. Southern blotting was carried using a TeloTTAGGG assay kit (Roche Applied Sciences) following the manufacturer's protocol with some modifications (Kimura et al., 2010).

Quantitative PCR assay for average telomere length measurement

Total genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instruction. The concentration and purity of genomic DNA were measured using nanodrop spectrophotometers (Thermo Scientific). Fresh genomic DNA

with high purity (an A260/A280 ratio of 1.8) was used for experiments. The integrity of genomic DNA was determined by gel electrophoresis. QPCR was performed as previously described (Cawthon, 2002) with further modifications (Jodczyk et al., 2015). Briefly, two qPCR reactions were carried using either primers for single copy reference gene (albumin) or telomeres at a final concentration of 900nM (See Supplementary Table 2 for primer sequences). In each reaction, a standard curve was made by serially diluting one reference DNA by 2 fold ranging from 1.56 ng to 50 ng in order to ensure the PCR efficiency of 90-100% with the linear correlation coefficient greater than 0.98 for each independent experiment. 5ng of sample genomic DNA was added in a 384 well plate with a total reaction volume of 15uL. Each reaction was performed in duplicate and duplicate CT values were averaged and then used to calculate for relative telomere copy number to single gene copy number (T/S) ratio.

Quantification of Dendritic Arborization

Neuronal cultures were immunostained for Tuj1 in order to identify mature neurons and visualize entire cells. The stained cells were then imaged at 20x and dendritic arbors of individual cells were traced using the Simple Neurite Tracer plugin for ImageJ. The number of process ends per cell were counted using the Cell Counter plugin for ImageJ. Only mature neurons—identifiable by their thin processes and condensed somas—were used for analysis. The number of process ends per cell are presented as mean ends per cell +/- SEM. Means were compared using the Student's T-Test for data with two groups. A p value <0.05 was used as the cutoff for significance.

RNA expression profiling

RNA purification was performed with Qiagen RNAeasy kit following the manufactures's instruction. Libraries were prepared according to the manufacturers guidelines using The TruSeq V2 kit (Illumina). Microarray profiling was performed with Affymetrix Human HG-U133 2.0 Plus arrays as described⁸⁵. For RNA sequencing, the datasets were mapped with RASER and HISAT2. The reads were counted per gene. Genes were defined by the exon union from the hg19 ensembl annotations. The function of DESeq in DESeq2 package was used to first normalize the gene read counts data and then identified the differentially expressed genes. The MA plot was generated with the function of plotMA in DESeq2 package. Q-value of 0.05 is regarded as the stringent cutoff of calling DEGs while p-value less than 0.05 is regarded as the low stringency cutoff. For the meta-chromosome plot of DEGs, all the chromosomes (except chromosome Y) were first divided equally into 20bins with different length, and then the number of DEGs in each bin was counted. GO analysis was performed using DAVID.

Analysis of 5hydroxymethyl-cytosine

5hmC capture (hmC-Seal)

Chemical labeling-based 5hmC enrichment was described previously⁸⁶. In brief, five microgram of genomic DNA was sonicated to 100-500 bp, and then mixed with 100 μ l reaction buffer (50 mM HEPES at pH 8.0, 25 mM $MgCl_2$, 250 μ M UDP-6- N_3 -Glu and 2.25 μ M wild-type β -glucosyltransferase (β -GT)). Reactions were incubated at 37°C for 1 hour, and DNA substrates were purified by Qiagen DNA purification kit. 150 μ M dibenzocyclooctyne modified biotin were mixed with β -GT-modified DNA. The labeling

reaction was performed at 37°C for 2 hours. The biotin-labeled DNA was then enriched by Streptavidin-coupled Dynabeads (Dynabeads MyOne™ Streptavidin T1, Life Technologies) and purified by Qiagen DNA purification kit. The quantity and quality of purified DNA were analyzed by Qubit 3.0 Fluorometer (Invitrogen) and Agilent 2100 BioAnalyzer using DNA high sensitivity analysis kits (Agilent Technologies).

MeDIP-seq

Methylated DNA Immunoprecipitation (MeDIP) experiments were performed according to the manufacturer's protocol (Active Motif) and described previously ⁸⁷. In brief, five microgram of genomic DNA were sonicated to 100-500 bp, and incubated with 5mC-specific antibody (Active Motif) at 4°C overnight. Enriched methylated DNA will be extensively washed and purified by Qiagen DNA purification kit. The quantity and quality of purified DNA were analyzed by Qubit 3.0 Fluorometer (Invitrogen) and Agilent Bioanalyzer using DNA high sensitivity analysis kits (Agilent Technologies).

Library Preparation and High-throughput Sequencing

Enriched DNA from MeDIP and hME-Seal will be subjected to library construction using the NEBNext ChIP-Seq Library Prep Reagent Set for Illumina according the manufacturer's protocol. In brief, 25 ng of input genomic DNA or experimental enriched DNA were utilized for each library construction. 150-300 bp DNA fragments were selected by AMPure XP Beads (Beckman Coulter) after the adapter ligation. An Agilent 2100 BioAnalyzer were used to quantify the amplified DNA, qPCR were applied to accurately quantify the library concentration. 20 pM diluted libraries were used for sequencing. 50-

cycle single-end sequencings will be performed. Image processing and sequence extraction were done using the standard Illumina Pipeline.

Analysis

Bowtie software was used to map the sequenced reads back to the human genome (hg19) with the parameter of allowing up to two mismatches. Only the uniquely mapped reads were then used to generate the piled-up genome coverage of methylation signals. The methylation signals were further normalized by per million mapped reads for following analysis. The human genome was then segmented into bins of 1kilo-base pairs, which allows for the identification of bins which shows most dramatic methylation signals differences between wild-type and mutant samples. The delta methylation signal of 0.2 per million reads was chosen as the cutoff of calling the Differential Methylation Regions. For the meta-chromosome plot, all the chromosomes (except chromosome Y) were divided equally into 20 bins with different length. We then summarized the total methylation signal within each bin and subtracted the signal between wild-type samples and mutant samples. The subtracted signal was then plotted as the meta-chromosomal plot of methylation differences.

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Chapter 4: Conclusions

Genomic function of MECP2

In this study, we used an isogenic iPSC model of MECP2 loss and clarified the function of MECP2 in a cell type specific manner. We derived multiple isogenic WT and MECP2 null iPSC lines from each of two independent patients who carried different mutations. This approach facilitated the study of MECP2 loss at several stages of development rather than any mutation or clone specific effects. We confirmed that these lines lack any erosion of X chromosome inactivation, as we demonstrated that the MECP2 locus remained silent even after extensive passaging and differentiation of our lines. We focused our studies on the GABAergic interneuron lineage because loss of MECP2 in this neuronal subtype has been shown to lead to severe Rett-like phenotypes, and dysfunction of these interneurons are seen in several neurological and psychiatric illnesses^{1,2} although non-cell-autonomous effects of MECP2 loss in astrocytes have also been reported^{3,4}.

To identify the molecular consequences of loss of MECP2, we first analyzed the global transcriptome of undifferentiated iPSCs, NPCs and neurons that either expressed a WT or mutant version of MECP2. The results of this analysis revealed a substantial number of differentially expressed genes in the absence of MECP2, with a greater number of differentially expressed genes at the neuronal stage of development. This suggests that the consequences of MECP2 loss may be compounded over development. Furthermore, the location of differentially expressed genes showed significant enrichment toward subtelomeric locations—the regions of the genome that are adjacent to the telomeres at the ends of chromosomes. Additionally, we observed a very strong enrichment for DhMRs

at subtelomeric domains in MECP2 null iPSCs, despite the fact that these domains do not appear to be enriched for 5hmC or show differential density of CpGs versus the rest of the genome in WT cells. It is important to note that the analysis presented were consistent across at least two clones from each of the two patients with different mutations. Importantly, there was a significant overlap in the location of the differentially expressed genes and the differentially methylated regions, suggesting a connection between these changes.

Several points remain to be addressed to further clarify the role of MECP2 in genomic function. Going forward, to confirm whether any of these gene expression changes are directly due to MECP2 loss, we could take MECP2 null cells and perform rescue experiments whereby MECP is ectopically overexpressed by lentiviral infection. If rescuing MECP2 expression ameliorates the effects on subtelomeric gene expression, that would indicate that MECP2 plays a dynamic role in regulating gene expression in these domains. The loss of 5hmC in iPSCs lacking MECP2 could have been due to either a failure to generate new methylation marks or a failure stabilize the methylation marks that had already been established. We speculate as a methylated DNA binding protein, MECP2 acts to stabilize these marks. If true, the reduction of 5hmC marks we observed would be due to the loss of MECP2-mediated stabilization of 5mc marks, leading to a diminished pool of 5mC marks for the TET enzymes to convert into 5hmc. To test this hypothesis, we could perform DNA 5mC methylation profiling (e.g. MeDIP) to analyze the presence of 5mC across the genome of our iPSCs, NPCs, and neurons. In fact, the same pool of genomic DNA previously isolated for 5hmC profiling can also be used for 5mC

profiling. By comparing the 5hmC and 5mC pattern in these various contexts, we could gain valuable mechanistic insights about the role for MECP2 in regulation of both 5mC and/or 5hmC. On the other hand, we could test whether MECP2 directly regulates 5hmC stability rather than regulating its formation by performing 5hmC profiling in an acute knock-down model. If acutely depleting MECP2 with RNAi leads to a loss of 5hmC marks, it would suggest that MECP2 stabilizes this mark, as opposed to facilitating its generation. Furthermore, we can take MECP2 null cells again and perform rescue experiments by overexpressing MECP2 from a lentiviral construct. We expect that rescue of MECP2 expression will ameliorate the loss of 5hmC at subtelomeric domains. This result would suggest MECP2 plays a dynamic role in stabilizing 5hmC marks as opposed to preventing their generation.

Lastly, we can ask whether the methylation differences caused by loss of MECP2 coincide with changes in chromatin accessibility at these genomic loci. We speculate that in the absence of MECP2, the loss of 5hmC leads to decondensation of chromatin, allowing transcriptional machinery to access the genes in the subtelomeric domains. To determine whether loss of MECP2 leads to an increase or decrease in chromatin accessibility, we can utilize the Assay for Transposase Accessible Chromatin (ATAC-seq), a high throughput method developed to ascertain the relative level of compaction across the genome with high sensitivity²². This would allow us to identify if methylation differences at discrete loci coincide with changes in chromatin structure and gene expression. These potential experiments would help us develop a better understanding of the genome-wide landscape of DNA methylations and their association with mRNA as

well as chromatin structure in relation with subtelomeres and telomeres in defined cell types.

Dysfunction of subtelomeres/telomeres and Rett syndrome

We also carried out a study of telomere length in relation to MECP2 expression. A previous study showed that a reporter gene inserted into the subtelomere has its expression silenced in a telomere length dependent manner; the longer the telomere, the greater the silencing⁵. Others showed that this effect can influence genes over 10 Mb from the end of a chromosome⁶. Our data suggest that iPSCs, NPCs and neurons lacking MECP2 have shorter telomeres as measured by qPCR in comparison to their isogenic WT counterparts. We have also observed an even more significant effect after acute MECP2-knockdown in WT NPCs and iPSC-derived MECP2-null neurons. To further uncover the role of MECP2 in the regulation of telomeres, we also looked at expression of TERRA, a long non-coding RNA that is known to significantly affect telomere structure and length by competing for telomeres in the telomerase complex⁷. In MECP2 null clones and after knockdown of MECP2 in WT NPCs and neurons, we observed strong induction of several TERRA transcripts. These results further demonstrate that MECP2 could regulate these loci in various cell types.

There are two competing hypotheses to explain the epigenetic and transcriptional changes that occur downstream of MECP2 loss: either MECP2 loss destabilizes subtelomeric genes, which then leads to shortened telomeres, or changes in telomeres drive the disruption in subtelomeric gene expression. To test these two hypotheses we

could acutely knock-down MECP2 in NPCs and neurons, and then measure DNA methylation, subtelomeric gene and TERRA expression, and telomere length at several different time points after knockdown. We predict that 1) loss of MECP2 destabilizes 5mc and/or 5hmC marks at the end of chromosomes, 2) leading to increased expression of subtelomeric genes and TERRAs, 3) which will result in shortening of telomeres. These time-course experiments would help shed light on the mechanism through which MECP2 regulates subtelomeric methylation, gene expression, and telomere length.

Given the fact that TERRA is transcribed from subtelomeric regions and in turn regulates telomere length, we predict that it will form the critical nexus point of all of the changes we have observed at the ends of chromosomes in cells lacking MECP2. A recent study from the Blasco lab demonstrated that TERRA can be silenced with a commercially available RNAi approach (LNA-RNA)⁸. We can use this reagent to block TERRA induction both in the presence and absence of MECP2 (both chronic and acute) in iPSCs, NPCs, and neurons and assay for telomere length and subtelomeric gene expression by qPCR. These experiments could therefore determine whether TERRA is the key molecular link between subtelomeric dysregulation and telomere shortening.

Our study took advantage of *in vitro* defined tools to explore the consequences of telomere length on gene expression specifically at subtelomeres. Thus far, we have made observations implicating MECP2 in telomeric and subtelomeric regulation in iPSCs, NPCs and neurons. Cells made in the absence of MECP2 show shorter telomeres, induction of subtelomeric genes including TERRA, and defective DNA methylation specifically in

subtelomeric domains. The additional experiments described above will further the role of MECP2 in gene regulation, DNA methylation and chromatin organization at subtelomeres and telomeres. Taken together, these findings will help advance our understanding of the disease mechanism of Rett syndrome and may help facilitate the discovery of new therapeutic approaches for this devastating disease.

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